Study and exploitation of varietal diversity for agroclimatic adaptation and nutritional content improvement in *Capsicum* spp.

PhD dissertation by

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VALENCIA, OCTOBER 2019

Agradecimentos

"Chegamos ao fim da canção…" – Ornatos Violeta

A canção durou cinco longos anos. Resta agora agradecer àqueles que me ajudaram a chegar até aqui e fizeram deste trabalho uma experiência enriquecedora, amena e cheia de boas memórias.

A mis directores, Adrián, Ana y Santi. Muchísimas gracias por, primero, aceptarme como discípulo o doctorando o como se diga, y, segundo, por desmontar en fascículos fáciles de montar lo que a veces parecía no dejarse entender. Gracias por la paciencia y por acogerme tan bien.

Al lab *Capsicum* y otros géneros. Gracias por acogerme, por los momentos compartidos antes, durante y después del trabajo y, más recientemente, gracias por suplantar mi ausencia para que pudiera escribir lo que viene a continuación. Gracias por contribuir a mi formación laboral y personal.

À Jumpy. Não podia deixar de mencionar aquela que foi um dos maiores pilares desta experiência. Apesar de já ter chegado na fase final, os seus conselhos, brincadeiras e personalidade tornaram tudo ainda mais especial. Obrigado.

À minha mãe, pai e irmã que, apesar de não terem enchido uma única caixa de pontas, não terem revelado um gel sequer ou nem sequer saber a diferença entre uma variedade tradicional e um híbrido comercial, merecem um lugar de destaque neste espaço. Muito obrigado por terem abdicado de tanto só para que eu pudesse ter o melhor.

À Elena. Muito obrigado por estares sempre ao meu lado, e tornar até as enfadonhas jornadas intensivas de trabalho numa experiência divertida. Obrigado por teres sempre algo positivo a dizer, não podia ter tido melhor sorte. 50.400.000,00 obrigados à melhor pessoa do mundo, à melhor tradutora do mundo, à melhor cúmplice e à melhor amiga do mundo.

"Fazes que fazes ou pões sementes a crescer…" – Sérgio Godinho

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Abstract |

Agriculture will face many challenges in the next generations, especially those related to food security and sustainability. In the advent of climate change, effective strategies to boost food production, without jeopardising the environment, are of paramount importance. Regarding that, peppers are one of the most relevant vegetables and part of the gastronomic and cultural heritage of many cultures. Likewise, peppers have a significant footprint on the environment, with almost four million hectares dedicated to their production. Hence, improving pepper varieties productivity, resistances and adaptation to low input conditions, would have a positive impact on food security and agricultural sustainability. In order to improve any crop, breeders require genetic diversity. Fortunately, *Capsicum* is remarkably diverse. Among the cultivated species, *C. annuum* is the most diverse and economically important taxon, and Spain is a relevant centre of diversity, where centuries of cultivation led to the bearing of a plethora of ecotypes adapted to a wide range of conditions. However, these materials still lack of proper typification and are at risk of disappearing.

Thus, the main goals herein were: i) to use both phenomics and genomic tools to characterize *Capsicum* spp. germplasm, with particular emphasis on Spanish *C. annuum* landraces, ecotypes and heirlooms. And ii) to characterize their nutritional value and their adaptation to abiotic stress conditions with the goal of exploiting the genotype per environment interaction as a mean to select elite materials for high productivity, nutritional value and adaptation to stress conditions. To achieve these goals, we performed the following works.

In this regard, conventional descriptors and digital parameters provide a vital help towards germplasm characterization. Herein we used conventional and digital descriptors to characterize a collection of the most relevant landraces from the Spanish centre of diversity, in order to assess the diversity and to test the discriminating ability of said methods. A considerable variation was found for both conventional and digital methods, even within closely related groups. However, digital phenotyping enabled a more powerful intra-varietal and inter-varietal separation, compared to conventional descriptors. This was particularly evident for closely related varieties. We conclude by selecting a subset of 4 conventional and 13 digital traits which enable to distinguish among closely related *C. annuum* accessions, explaining 81.81% of total variance found by PCA. Finally, fruit traits explained the highest percentage of variance for our collection. These findings will be useful to the recovery of heirloom peppers and will boost germplasm characterization and management in seed banks.

Landraces are fruit of a long selection process moulded by environmental conditions, and maintained through open-pollination and recombination events, that conferred these materials their inherently wide genetic diversity and resilience. The study of both

morphologic and genetic diversity is an important practice in order to select the most interesting materials to be used in breeding programs. Thus, some of the most known landraces can be found in Mexico. These ancient heirlooms, known as *criollos*, encompass a remarkable aptitude to be used as pre-breeding materials or as a diverse population with higher resilience to climate change conditions. Hence, we studied the phenotypic and genotypic diversity within these materials and the open-pollination effect on the fixation of morphological characteristics and on the genetic fixation by characterizing two 'chile Ancho' lines and their progenies, using IPGRI descriptors and SSR molecular markers. Morphological analysis, given by PCA, showed similar levels of agronomic and morphological uniformity within the two families, indicating that standard phenotype is maintained despite the lack of controlled pollination. Plant and fruit traits explained most of the variation within our collection. In addition, high levels of heterozygosity were found, although, progenies showed similar or lower level than those from progenitors, suggesting that open-pollinated program is efficient in terms of reaching enough agronomic uniformity in *criollo* peppers, while preserving a certain degree of genetic diversity, of paramount importance for the adaptation to climate change, particularly for poor and low input agricultural systems.

Spain detains a remarkable amount of traditional varieties that still lack of proper typification and are at risk of disappearing. Germplasm genetic studies provide vital information for crop breeding and contribute for biodiversity conservation. However, they are highly dependent on the availability of informative genetic tools. In that regard, pepper genomics lag behind those of other important *Solanaceae*. Herein we used high-throughput genotyping-by-sequencing to study a collection of *Capsicum* spp., encompassing a representative collection of Spanish heirlooms, to shed light into the Spanish landraces phylogenetic relationships and to evaluate their molecular diversity and population structure. Sequencing generated 6,766,231 high quality read tags, of which 41% successfully aligned to the reference genome. In addition, SNP calling yielded 4,083 highly informative SNPs which were used for genetic diversity and phylogeny analysis. Thus, *C. annuum*, *C. baccatum* and *C. chinense* accessions were successfully separated by all methods. Our population was divided into seven clusters where *C. frutescens* accessions clustered together with *C. chinense* whereas *C. annuum var. glabriusculum* accessions were spread into two distinct genetic pools. Furthermore, fruit traits and region of origin were the main factors defining population structure. Spanish and Mexican accessions showed a close phylogenetic relationship, supporting the hypothesis that the first arose from a main genetic flow from the latter. Finally, Tajima's D statistic values were consistent with positive selection in the *C. annuum* clusters related to domestication or selection towards traits of interest. These findings provide relevant information on the origin and relationships of Spanish landraces and for future association mapping studies in pepper. Ultimately, we provide important tools regarding pepper breeding, and validate the applicability of genotyping-by-sequencing in pepper genetic studies.

Peppers are known as one of the most nutritious vegetables in the world. However, breeding programs have forsaken health-promoting compounds in spite of productivity and resistance to biotic stresses. However, increasing interest for the "taste of the past" and the need to adapt to climate change conditions are contributing to the reintroduction of traditional varieties. On that matter, *C. baccatum* represents a remarkable genetic pool that provides opportunity to select superior individuals to be used in breeding programs for improved bioactive compounds content and resilience to climate change materials. Hence, we characterized a collection of *C. baccatum* and *C. annuum* materials for their ascorbic acid, protein and mineral profile, under greenhouse and open-field conditions, as a first step towards the selection of highly adapted to Mediterranean conditions *C. baccatum* varieties with satisfactory internal quality. Cultivation system had a major effect controlling fruit's nutrient profile, particularly for *C. baccatum*. Thus, *C. annuum* controls presented higher concentrations for most compounds under both conditions. The good performance of *C. baccatum* accessions shows that there are opportunities to breed materials adapted to the Mediterranean conditions and with interesting properties, especially under open-field conditions. Finally, under our conditions, a serving of pepper cultivated could provide between 70% and 120% of the recommended dietary allowance for ascorbic acid and between 10% and 60% for minerals.

Improving pepper varieties for their uptake and use of phosphorus would significantly reduce the need for fertilizer applications and would have a significant impact on poor and low input agricultural systems. This would be particularly useful for phosphorus because the lack of this mineral is a major constraint to food production all around the world. Hence, we characterized the main root adaptations, of a *Capsicum* spp. collection, to low phosphorus inputs. We report herein a wide range of variation regarding morphological traits and phosphorus use efficiency. Overall, stress conditions lead to significant reduction of biomass in all genotypes. In addition, stress treatment stimulated lateral root length and root hairs growth; both traits are linked to enhanced phosphorus acquisition. Furthermore, as expected, under phosphorus starvation, concentration of this mineral in plant tissues decreased significantly. This response was notoriously higher in the roots, demonstrating high ability to mobilise accumulated phosphorus to favour other plant organs. Accessions demonstrated a wide range of responses among our collection. Thus, providing evidence that within the *Capsicum* genus there is usable variability for phosphorus use efficiency for breeding programs for low input adaptation, enabling the combination of several favourable traits or behaviours in a single genotype, which in return can be a more effective solution towards the improvement of pepper's resilience to low input phosphorus conditions.

We provide herein useful information for *Capsicum* genetic resources management, with particular emphasis on Spanish germplasm. We aimed at providing tools and knowledge to contribute to typification, promotion and conservation of the genetic and morphologic diversity of *Capsicum*. Likewise, we sought to provide relevant insights

into the state of the art regarding *Capsicum* breeding in order to stimulate the development of pepper varieties with enhanced productivity, internal content and resilience to climate change.

Resumen |

En las próximas generaciones, la agricultura se deberá enfrentar a grandes desafíos, especialmente aquellos relacionados con la seguridad alimentaria y la sostenibilidad agrícola. Con la llegada del cambio climático resulta de suma importancia la búsqueda de estrategias efectivas para impulsar la producción de alimentos sin poner en riesgo el medio ambiente. A este respecto, el pimiento es una de las hortalizas más relevantes y parte del patrimonio gastronómico y cultural de diversas civilizaciones. Asimismo, los pimientos generan una huella significativa en el medio ambiente, con casi cuatro millones de hectáreas dedicadas a su producción. Por lo tanto, mejorar las variedades de pimiento para rendimiento, resistencias y adaptación a condiciones de bajos insumos, tendría un impacto significativo en la seguridad alimentaria y la sostenibilidad agrícola. Para la mejora de cualquier cultivo, un requisito indispensable es la variabilidad genética. Afortunadamente, *Capsicum* es muy diversa. Entre las especies cultivadas, *C. annuum* es la especie más diversa y económicamente importante, y España es un centro secundario de diversidad altamente relevante. De este modo, siglos de cultivo han llevado a una gran cantidad de ecotipos españoles adaptados a una amplia gama de condiciones. A pesar de ello, estos materiales carecen de una caracterización y tipificación adecuadas, tareas de importancia primordial en el manejo del germoplasma.

En este sentido, los objetivos principales de esta tesis fueron: i) la caracterización fenómica y genómica de germoplasma de *Capsicum* sp., con especial énfasis en las variedades locales y ecotipos españoles de *C. annuum*. Y ii) la caracterización de su valor nutricional y su adaptación a las condiciones de estrés abiótico con el objetivo de explotar la interacción genotipo por ambiente como un medio para seleccionar materiales de élite para una alta productividad, valor nutricional y adaptación a las condiciones de estrés. Para ello se han realizado diferentes trabajos que se resumen a continuación.

Para la caracterización morfológica, los descriptores convencionales y la fenómica digital proporcionan una ayuda vital para la caracterización del germoplasma. En este trabajo hemos utilizado descriptores convencionales y digitales para caracterizar una colección representativa de las variedades locales más relevantes del centro de diversidad español, con el fin de evaluar la variabilidad dentro del germoplasma de élite español y probar la eficiencia de esos métodos para diferenciar los materiales estrechamente relacionados. Los resultados de este estudio muestran una considerable variación, para los descriptores convencionales y digitales, incluso dentro de grupos estrechamente relacionados; aunque el fenotipado digital fue capaz de separar los grupos varietales en un mayor número de categorías. Del mismo modo, el fenotipado digital permitió una separación intravarietal más potente en comparación con los descriptores convencionales. Finalmente, se seleccionó un subconjunto de 4 descriptores convencionales y 13 descriptores digitales para distinguir entre accesiones de *C. annuum*

estrechamente relacionadas, explicando el 81.81% de la varianza total detectada por ACP. Otra conclusión importante de este trabajo es que los rasgos del fruto son los más relevantes para la separación entre variedades y explican el mayor porcentaje de variación para nuestra colección. Estos hallazgos serán útiles para la recuperación de las variedades de polinización abierta e impulsarán la caracterización y el manejo del germoplasma en los bancos de semillas.

Las variedades tradicionales son el resultado de un largo proceso de selección, moldeado por las condiciones ambientales y mantenido mediante polinización abierta y eventos recombinantes, que confiere a estos materiales su característica variabilidad genética y elevada resiliencia. El estudio de diversidad morfológica y genética es una práctica importante que posibilita la selección de los materiales más interesantes para ser introducidos en programas de mejora. Asimismo, muchas de las variedades tradicionales más apreciadas se pueden encontrar en México. Estos materiales ancestrales, también conocidos como *criollos*, poseen una aptitud extraordinaria para ser usados como materiales de pre-mejora o, igualmente, como una población diversa y con una alta resiliencia a las condiciones del cambio climático. Por lo tanto, nos propusimos estudiar la diversidad fenotípica y genotípica de estos materiales, así como el efecto de la polinización abierta en la fijación de las características morfológicas y en la fijación genética. Para ello, caracterizamos dos líneas de 'chile Ancho', conjuntamente con sus correspondientes descendencias, usando descriptores IPGRI y una batería de marcadores moleculares de tipo SSR. La caracterización morfológica mostró, mediante el ACP, niveles similares de uniformidad para las dos familias consideradas, indicando que el morfotipo estándar de la variedad es mantenido, a pesar de la ausencia de control de la polinización en este sistema de cultivo. Los caracteres de planta y fruto explicaron el mayor porcentaje de variación dentro de la colección considerada aquí. Además, se observaron altos niveles de heterocigosidad; sin embargo, la progenie mostró niveles similares o inferiores a aquellos mostrados por las líneas parentales, sugiriendo que el sistema de reproducción mediante polinización abierta es eficiente en términos de alcanzar uniformidad agronómica y a la vez preservando un cierto grado de diversidad genética, un hecho de enorme relevancia para la adaptación a las condiciones de cambio climático, especialmente en sistemas agrícolas pobres y de bajos insumos.

España posee una impresionante cantidad de variedades tradicionales, sin embargo, muchas de ellas carecen aún de una tipificación adecuada, siendo que muchas se encuentran en riesgo de desaparecer. En ese sentido, los estudios genéticos del germoplasma existente aportan información de gran utilidad, tanto para la mejora de los cultivos como para la conservación de los recursos fitogenéticos. Sin embargo, estos estudios dependen en gran medida de la disponibilidad de herramientas genómicas informativas. Desafortunadamente, los estudios genómicos en pimiento están por detrás de los de otras solanáceas importantes. En la presente tesis doctoral se utilizó el genotipado por secuenciación de alto rendimiento (GBS) para estudiar una colección de

Capsicum spp., que abarca un conjunto de las variedades tradicionales españolas más representativas, con el fin de arrojar luz sobre las relaciones filogenéticas de las variedades locales y evaluar su diversidad molecular y estructura de la población. La secuenciación generó 6,766,231 *read tags* de alta calidad, de las cuales el 40.7% se alinearon exitosamente con el genoma de referencia. Además, *SNP calling* produjo 4,083 SNPs segregantes altamente informativos. La diversidad genética se analizó mediante ACP, Análisis Discriminante de Componentes Principales y estudios de filogenia. Las especies *C. annuum*, *C. baccatum* y *C. chinense* fueron separadas con éxito por todos los métodos. Nuestra población se dividió en siete grupos, donde las accesiones de *C. frutescens* se agruparon junto con *C. chinense*. *Capsicum annuum var. gabriusculum* se dividió en dos grupos genéticos distintos, mientras que las accesiones europeas estaban estrechamente relacionadas. Además, las características de fruto y el origen fueron los principales factores que determinaron la separación de la accesión y la estructura genética. Los estudios de filogenia también mostraron una estrecha relación entre las accesiones española y mexicana, apoyando la hipótesis de que la primera surgió de un flujo genético principal de esta última. Los valores del estadístico Tajima D fueron consistentes con la selección positiva en los grupos de *C. annuum* relacionados con la domesticación o la selección hacia rasgos de interés. Así, en el presente trabajo se presenta información completa y relevante sobre el origen y las relaciones de las variedades locales españolas, lo que será útil y relevante para futuros estudios de mapeo por asociación en pimiento. Esta es la primera vez que se utiliza el GBS para estudiar una colección tan notable de variedades locales españolas generando a su vez una cantidad considerable de SNPs altamente informativos.

Los pimientos son conocidos como uno de los vegetales más nutritivos del mundo; sin embargo, los programas de mejora han dejado de lado los compuestos bioactivos y el contenido en minerales, entre otras sustancias promotoras de la salud. A pesar de ello, el creciente interés por el "sabor de antes" por parte de los consumidores y el desafío de adaptarse a las condiciones del cambio climático están contribuyendo a la mejora y reintroducción de materiales tradicionales. En ese sentido, *C. baccatum* surge como una importante fuente de variabilidad genética y proporcionando a los investigadores la oportunidad de seleccionar aquellos individuos élite para ser introducidos en programas de mejora para el contenido en compuestos bioactivos y resilientes a las condiciones del cambio climático. Por ello, en este trabajo, decidimos caracterizar una colección de *C. baccatum* y *C. annuum* en cuanto a su contenido en ácido ascórbico, proteína y minerales bajo dos sistemas de cultivo, invernadero y aire libre, con el fin de seleccionar individuos con una alta adaptación al clima mediterráneo y un contenido en compuestos bioactivos satisfactorio. Se observó una gran influencia del sistema de cultivo sobre el contenido nutricional de los frutos de pimiento, esta influencia se ha visto superior en *C. baccatum*. Además, las concentraciones de estos compuestos se vieron favorecidas bajo cultivo al aire libre. Asimismo, las accessiones control de *C. annuum* mostraron concentraciones superiores para la mayoría de compuestos analizados para los dos

ambientes. Por otro lado, la buena respuesta de las accesiones de *C. baccatum* demuestra que existe la posibilidad de mejorar estos materiales de cara a obtener individuos altamente adaptados a las condiciones mediterráneas y un alto contenido en compuestos bioactivos, particularmente para las condiciones de cultivo al aire libre. Por último, bajo nuestras condiciones, una ración de pimiento puede aportar entre un 70% y un 120% de la dosis diaria recomendada de ácido ascórbico y entre un 10% y un 60% de la dosis recomendada de minerales.

La mejora de las variedades de pimiento para la absorción y uso del fósforo es de suma importancia en la tarea actual de reducir significativamente la necesidad de aplicaciones de fertilizantes. Además, tendría un impacto significativo en el rendimiento de los sistemas agrícolas pobres y de bajos insumos del mundo subdesarrollado y en desarrollo. Esto sería particularmente útil para el fósforo porque la falta de este mineral es una limitación importante para la producción de alimentos en todo el mundo. Por lo tanto, el objetivo de este trabajo fue caracterizar una colección de pimiento frente a bajos insumos de fósforo. En este documento presentamos un amplio rango de variación con respecto a los rasgos morfológicos y la eficiencia del uso del fósforo. En general, las condiciones de estrés condujeron a una reducción significativa de la biomasa en todos los genotipos. Además, el tratamiento de bajo fósforo estimuló el desarrollo de la raíz lateral y de pelos radiculares en las raíces, ambos rasgos relacionados con la adquisición mejorada de fósforo. Además, como se esperaba, bajo la inanición de fósforo, la concentración de este mineral en los tejidos vegetales disminuyó significativamente. Esta respuesta fue notoriamente superior en las raíces, mostrando la capacidad de movilizar el fósforo acumulado hacia otros órganos con el fin de favorecer su desarrollo. Esto proporciona evidencias de la existencia de variabilidad dentro de *Capsicum* para la eficiencia del uso del fósforo susceptible de ser utilizada en los programas de mejora para la adaptación del cultivo a bajos insumos, posibilitando igualmente la combinación de varios rasgos de interés en un mismo genotipo, lo que podría resultar más eficiente en la mejora del pimiento frente a bajos insumos de fósforo.

La presente tesis doctoral presenta información útil para el manejo de los recursos genéticos de *Capsicum*, con especial énfasis en el germoplasma español. Nuestro objetivo fue proporcionar herramientas y conocimiento que puedan contribuir a la tipificación, promoción y conservación de la diversidad genética y morfológica del género *Capsicum*. Del mismo modo, este trabajo proporciona información relevante sobre las bases de la mejora de *Capsicum* para estimular el desarrollo de variedades de pimiento con mayor productividad, contenido en compuestos bioactivos y resiliencia al cambio climático.

Resum |

En les pròximes generacions, l'agricultura s'haurà d'enfrontar a grans desafiaments, especialment aquells relacionats amb la seguretat alimentària i la sostenibilitat agrícola. Amb l'arribada del canvi climàtic resulta de summa importància la cerca d'estratègies efectives per a impulsar la producció d'aliments sense posar en risc el medi ambient. Referent a això, el pimentó és una de les hortalisses més rellevants i part del patrimoni gastronòmic i cultural de diverses civilitzacions. Així mateix, els pimentons generen una petjada significativa en el medi ambient, amb quasi quatre milions d'hectàrees dedicades a la seua producció. Per tant, millorar les varietats de pimentó per a rendiment, resistències i adaptació a condicions de baixos inputs, tindria un impacte significatiu en la seguretat alimentària i la sostenibilitat agrícola. Per a la millora de qualsevol cultiu, un requisit indispensable és la variabilitat genètica. Afortunadament, *Capsicum* és molt diversa. Entre les espècies cultivades, *C. annuum* és l'espècie més diversa i econòmicament important, i Espanya és un centre secundari de diversitat altament rellevant. D'aquesta manera, segles de cultiu han portat a una gran quantitat d'ecotips espanyols adaptats a una àmplia gamma de condicions. Malgrat això, aquests materials manquen d'una caracterització i tipificació adequades, tasques d'importància primordial en el maneig del germoplasma.

En aquest sentit, els objectius principals d'aquesta tesi són: i) la caracterització fenòmica i genòmica de germoplasma de *Capsicum* sp., amb especial interès en les diverses varietats locals i ecotipus de *C. anuum* espanyols. I ii) la caracterització del seu valor nutricional i la seva adaptació a les condicions d'estrès abiòtic amb l'objectiu d'explotar la interacció genotipus per l'entorn com un mitjà per a seleccionar materials d'elit per a una alta productivitat, valor nutricional i adaptació a les condicions d'estrès. Per aconseguir-ho es van realitzar diferents treballs que s'expliquen a continuació.

En aquest sentit, els descriptors convencionals i la fenòmica digital proporcionen una ajuda vital per a la caracterització del germoplasma. En aquest treball hem utilitzat descriptors convencionals i digitals per a caracteritzar una col·lecció representativa de les varietats locals més rellevants del centre de diversitat espanyol, amb la finalitat d'avaluar la variabilitat dins del germoplasma d'elit espanyol i provar l'eficiència d'aqueixos mètodes per a diferenciar els materials estretament relacionats. Els resultats d'aquest estudi mostren una considerable variació, per als descriptors convencionals i digitals, fins i tot dins de grups estretament relacionats; encara que el fenotipat digital va ser capaç de separar els grups varietals en un major nombre de categories. De la mateixa manera, el fenotipat digital va permetre una separació intravarietal més potent en comparació amb els descriptors convencionals. Finalment, es va seleccionar un subconjunt de 4 descriptors convencionals i 13 descriptors digitals per a distingir entre accessions de *C. annuum* estretament relacionades, explicant el 81.81% de la variància total detectada per ACP. Una altra conclusió important d'aquest treball és que els trets

del fruit són els més rellevants per a la separació entre varietats i expliquen el major percentatge de variació per a la nostra col·lecció. Aquestes troballes seran útils per a la recuperació de les varietats de pol·linització oberta i impulsaran la caracterització i el maneig del germoplasma en els bancs de llavors.

Les varietats tradicionals són el resultat d'un llarg procés de selecció, modelat per les condicions ambientals i mantingut mitjançant pol·linització oberta i esdeveniments recombinants, que confereix a aquests materials la seua característica variabilitat genètica i elevada resiliència. L'estudi de diversitat morfològica i genètica és una pràctica important que possibilita la selecció dels materials mes interessants per a ser introduïts en programes de millora. Així mateix, moltes de les varietats tradicionals més benvolgudes es poden trobar a Mèxic. Aquests materials ancestrals, també coneguts com a *criolls*, posseeixen una aptitud extraordinària per a ser usats com a materials de pre-millora o, igualment, com una població diversa i amb un alta resiliència a les condicions del canvi climàtic. Per tant, ens vam proposar estudiar la diversitat fenotípica i genotípica d'aquests materials, així com l'efecte de la pol·linització oberta en la fixació de les característiques morfològiques i en la fixació genètica. Per a això, caracteritzem dues línies de 'chile Ancho', conjuntament amb les seues corresponents descendències, usant descriptors IPGRI i una bateria de marcadors moleculars de tipus SSR. La caracterització morfològica va mostrar, mitjançant el ACP, nivells similars d'uniformitat per a les dues famílies considerades, indicant que el morfotipus estàndard de la varietat és mantingut, malgrat l'absència de control de la pol·linització en aquest sistema de cultiu. Els caràcters de planta i fruit van explicar el major percentatge de variació dins de la col·lecció considerada ací. A més, es van observar alts nivells de heterozigositat; no obstant això, la progènie va mostrar nivells similars o inferiors a aquells mostrats per les línies parentals, sugerint que el sistema de reproducció mitjançant pol·linització oberta és eficient en termes d'aconseguir uniformitat agronòmica i alhora preservant un cert grau de diversitat genètica, un fet d'enorme rellevància per a l'adaptació a les condicions de canvi climàtic, especialment en sistemes agrícoles pobres i de baixos inputs.

Espanya posseeix una impressionant quantitat de varietats tradicionals; no obstant això, moltes d'elles manquen encara d'una tipificació adequada, sent que moltes es troben en risc de desaparéixer. En aqueix sentit, els estudis genètics del germoplasma existent aporten informació de gran utilitat, tant per a la millora dels cultius com per a la conservació dels recursos fitogenètics. No obstant això, aquests estudis depenen en gran manera de la disponibilitat d'eines genòmiques informatives. Desafortunadament, els estudis genòmics en pimentó estan per darrere dels d'altres solanáceas importants. En la present tesi doctoral es va utilitzar el genotipat per seqüenciació d'alt rendiment (GBS) per a estudiar una col·lecció de *Capsicum* spp., que abasta un conjunt de les varietats tradicionals espanyoles més representatives, amb la finalitat de llançar llum sobre les relacions filogenètiques de les varietats locals i avaluar la seua diversitat molecular i estructura de la població. La seqüenciació va generar 6,766,231 *read tags* d'alta qualitat,

de les quals el 40.7% es van alinear reeixidament amb el genoma de referència. A més, *SNP calling* va produir 4,083 SNPs segregants altament informatius. La diversitat genètica es va analitzar mitjançant ACP, Anàlisi Discriminant de Components Principals i estudis de filogènia. Les espècies *C. annuum*, *C. baccatum* i *C. chinense* van ser separades amb èxit per tots els mètodes. La nostra població es va dividir en set grups, on les accessions de *C. frutescens* es van agrupar juntament amb *C. chinense*. *Capsicum annuum* var. *gabriusculum* es va dividir en dos grups genètics diferents, mentre que les accessions europees estaven estretament relacionades. A més, les característiques i l'origen dels fruits van ser els principals factors que van determinar la separació de l'accessió i l'estructura genètica. Els estudis de filogènia també van mostrar una estreta relació entre les accessions espanyola i mexicana, donant suport a la hipòtesi que la primera va sorgir d'un flux genètic principal d'aquesta última. Això ho corroboren els valors del mètode estadístic Tajima D, que van ser consistents amb la selecció positiva en els grups de *C. annuum* relacionats amb la domesticació o la selecció cap a trets d'interés. Així, en el present treball es presenta informació completa i rellevant sobre l'origen i les relacions de les varietats locals espanyoles, la qual cosa serà útil i rellevant per a futurs estudis de mapatge per associació en pimentó. Aquesta és la primera vegada que s'utilitza el GBS per a estudiar una col·lecció tan notable de varietats locals espanyoles generant al seu torn una quantitat considerable de SNPs altament informatius.

Els pimentons són coneguts com un dels vegetals més nutritius del món; no obstant això, els programes de millora han deixat de costat els compostos bioactius i el contingut en minerals, entre altres substàncies promotores de la salut. Malgrat això, el creixent interés pel "sabor d'abans" per part dels consumidors i el desafiament d'adaptar-se a les condicions del canvi climàtic estan contribuint a la millora i reintroducció de materials tradicionals. En aqueix sentit, *C. baccatum* sorgeix com una important font de variabilitat genètica i proporcionant als investigadors l'oportunitat de seleccionar aquells individus elit per a ser introduïts en programes de millora per al contingut en compostos bioactius i resilients a les condicions del canvi climàtic. Per això, en aquest treball, decidim caracteritzar una col·lecció de *C. baccatum* i *C. annuum* quant al seu contingut en àcid ascòrbic, proteïna i minerals sota dos sistemes de cultiu, hivernacle i aire lliure, amb la finalitat de seleccionar individus amb una alta adaptació al clima mediterrani i un contingut en compostos bioactius satisfactori. Es va observar una gran influència del sistema de cultiu sobre el contingut nutricional dels fruits de pimentó, aquesta influència s'ha vist superior en *C. baccatum*. A més, les concentracions d'aquests compostos es van veure afavorides baix cultiu a l'aire lliure. Així mateix, les accessiones control de *C. annuum* van mostrar concentracions superiors per a la majoria de compostos analitzats per als dos ambients. D'altra banda, la bona resposta de les accessions de *C. baccatum* demostra que existeix la possibilitat de millorar aquests materials de cara a obtindre individus altament adaptats a les condicions mediterrànies i un alt contingut en compostos bioactius, particularment per a les condicions de cultiu a l'aire lliure. Per ultime, sota les nostres condicions, una ració de pimentó pot aportar entre un 70% i un 120% de la dosi diària recomanada d'acidifique ascòrbic i entre un 10% i un 60% de la dosi recomanada de minerals.

La millora de les varietats de pimentó per a l'absorció i ús del fòsfor és de summa importància en la tasca actual de reduir significativament la necessitat d'aplicacions de fertilitzants. A més, tindria un impacte significatiu en el rendiment dels sistemes agrícoles pobres i de baixos inputs del món subdesenvolupat i en desenvolupament. Això seria particularment útil per al fòsfor perquè la falta d'aquest mineral és una limitació important per a la producció d'aliments a tot el món. Per tant, l'objectiu d'aquest treball va ser caracteritzar una col·lecció de pimentó enfront de baixos inputs de fòsfor. En aquest document presentem un ampli rang de variació respecte als trets morfològics i l'eficiència de l'ús del fòsfor. En general, les condicions d'estrés van conduir a una reducció significativa de la biomassa en tots els genotips. A més, el tractament de baix fòsfor va estimular el desenvolupament de l'arrel lateral i de pèls radiculars en les arrels, tots dos trets relacionats amb l'adquisició millorada de fòsfor. A més, com s'esperava, sota la inanició de fòsfor, la concentració d'aquest mineral en els teixits vegetals va disminuir significativament. Aquesta resposta va ser notòriament superior en les arrels, mostrant la capacitat de mobilitzar el fòsfor acumulat cap a altres òrgans de manera que afavoreix el seu desenvolupament. Això proporciona evidències de l'existència de variabilitat dins de *Capsicum* per a l'eficiència de l'ús del fòsfor susceptible de ser utilitzada en els programes de millora per a l'adaptació del cultiu a baixos inputs, possibilitant igualment la combinació de diversos trets d'interés en un mateix genotip, la qual cosa podria resultar mes eficient en la millora del pimentó enfront de baixos inputs de fòsfor.

La present tesi doctoral presenta informació útil per al maneig dels recursos genètics de *Capsicum*, amb especial èmfasi en el germoplasma espanyol. El nostre objectiu va ser proporcionar eines i coneixement que puguen contribuir a la tipificació, promoció i conservació de la diversitat genètica i morfològica del gènere *Capsicum*. De la mateixa manera, aquest treball proporciona informació rellevant sobre les bases de la millora de *Capsicum* per a estimular el desenvolupament de varietats de pimentó amb major productivitat, contingut en compostos bioactius i resiliència al canvi climàtic.

Introduction |

1| Economic relevance of *Capsicum*

Peppers are the 7th most produced vegetable in the world only behind relevant vegetables such as tomato, onion, cucumber, cabbage, eggplant and carrot (FAO, 2019).

In 2016, 38.42 million tonnes (Mt) of pepper were produced in the world in a dedicated area of 3.74 million hectares (Mha), and yielding, on average, 10.27 t/ha. In addition, pepper's relevance has been increasing in the past decades and, consequently, its harvested area and production volume have increased during that same period. Thus, last available data from FAO shows an average increase of 0.72% and 3.06% per year for harvested area and production volume, respectively, since the year 2000. Likewise, average yield has been growing on an average of 2.01% a year, fruit of the adoption of the latest technological advances, such as acclimatised greenhouses, higher inputs, and introduction of improved varieties, particularly in developing countries (FAO, 2019).

Despite pepper's successful diffusion throughout the globe, an important fraction of its production is concentrated in Asia, totalizing 68.19% (26.19 Mt). The American continent is ranked second with 11.95% (4.59 Mt) of total production volume, followed closely by the African continent with 11.32% (4.35 Mt). Lastly, Europe represents 8.43% (3.24 Mt) of total production and Oceania a residual 0.12% (0.04 Mt). Regarding countries' individual contributions, China has the largest dedicated area (797298 ha) as well as the largest production volume (17.74 Mt), while the Netherlands and Belgium have the highest yields per ha (277.57 and 276.00 t/ha, respectively). Likewise, Spain is the 6th biggest world producer and detains the 10th highest yield in the world, with 1.09 Mt and 56.69 t/ha, respectively (Table 1).

Table 1 - Top ten ranking of countries with highest harvested area (ha), production volume (Mt) and yield (t/ha) for pepper (green + dry) (FAO 2019).

Rank	Country	H. area (ha)	Country	Production (Mt)	Country	Yield (t/ha)
1	China	797298	China	17.74	Netherlands	277.57
2	India	797029	Mexico	2.80	Belgium	276.00
3	Indonesia	260222	Turkey	2.47	UK	256.00
4	Mexico	202762	Indonesia	1.96	Germany	114.33
5	Ethiopia	190533	India	1.46	Finland	106.83
6	Nigeria	135751	**Spain**	**1.09**	Austria	86.59
7	Myanmar	110051	USA	0.92	Kuwait	81.41
8	Bangladesh	101972	Nigeria	0.81	Uruguay	66.61
9	Turkey	95708	Egypt	0.69	Chile	61.55
10	Thailand	93467	Algeria	0.61	**Spain**	**56.69**

In the year 2016, Spain's pepper production value achieved a total of €974.815,00, an important contribution to the country's economy, and a remarkable cipher that was higher than the average of the previous decade (€750.429,00) (MAPA, 2019). Andalusia is by far the autonomous community with the largest harvested area and production, representing 60.78% and 64.63%, respectively. In fact, most of its pepper cultivation is concentrated in just one province, Almeria, which represents 87.86% of the autonomous community total production and an impressive 56.79% of the country's total production. The Region of Murcia occupies the ranking's second position with 7.04% of total dedicated area and 12.23% of total production (Table 2).

Table 2 - Ranking of the 17 Spanish autonomous communities by their pepper production (t), and their corresponding percentage of the country's total production, harvested area (ha) and percentage of the country's total area for the year 2016 (MAPA 2019).

Rank	Autonomous communities	Production (t)	% of country's production	Harvested area (ha)	% of country's area
1	Andalusia	757893	64.63	11832	60.78
2	Region of Murcia	143447	12.23	1370	7.04
3	Galicia	65499	5.59	1202	6.17
4	Valencian Community	57020	4.86	819	4.21
5	Castilla-La Mancha	46428	3.96	1187	6.10
6	Community of Navarra	36268	3.09	1043	5.36
7	Extremadura	23886	2.04	572	2.94
8	Canary Islands	16072	1.37	232	1.19
9	Catalonia	6529	0.56	274	1.41
10	La Rioja	5902	0.50	202	1.04
11	Basque Country	4752	0.41	293	1.51
12	Castile and León	3317	0.28	151	0.78
13	Balearic Islands	2469	0.21	90	0.46
14	Aragon	2220	0.19	131	0.67
15	Principality of Asturias	478	0.04	51	0.26
16	Community of Madrid	347	0.03	12	0.06
17	Cantabria	112	0.01	7	0.04

2| Etymology of *Capsicum*

'Pepper', 'pimiento', 'chile', 'ají', 'paprika', 'rocoto' and 'tabasco' are some of the terms used to make reference to distinct forms of *Capsicum*, a genus characterized by a vast amount of variability (Bosland and Votava, 2012; DeWitt and Bosland, 1996). That being said, it is important to understand that the nomenclature used for this taxon does not comply by a standardised set of rules. Instead, historic, cultural and geographic-related criteria dictated how the distinct forms are designated in the respective domestication regions.

Before the arrival of the Spaniards to the Americas, natives had several names referring to *Capsicum*, a major ingredient in their diet. In Mexico, the Aztec civilisation named it *chilli,* in the extinct Nahuatl language. Nowadays, 'chile' is the used term in both Mexico and United States of America when referring to both the fruits and the plant, while 'chili' is used for a dish prepared with 'chile' and meat (Bosland and Votava, 2012). In Central and South America *Axí* was the predominant term before the arrival of the Spaniards, which originated from the also extinct Arawak language. *Axí* evolved to 'ají' in today's Spanish language and is still used in the Caribbean and most of South America to name the local pungent varieties (Andrews, 1995; APEGA et al., 2009). Other examples of designations are *Uchu*, a Quechuan word used by the Inca's empire, and *Huayca*, in the Aymara language spoken in some parts of the Inca's empire (Andrews, 1995; APEGA et al., 2009; Nuez et al., 2003).

The designation *pepper* appeared much later, during the first Spanish expeditions throughout American territories, and was a "misfortune" that stuck till this day (Bosland and Votava, 2012). Thus, *Capsicum* fruits were incorrectly named after *Piper nigrum* L. (*Piperaceae*), another plant altogether, which at the time had an incredible value as a spice. Just like black pepper, *Capsicum* fruits were used as a spice by natives and because of that Spaniards believed that both plants were related (Andrews, 1995; Bosland and Votava, 2012). After its introduction into the Old World and subsequent spread into almost all continents, new designations such as 'piri-piri', 'pimentão', 'pimentón', 'peperone', 'felfel' or 'paprika', arose to name *Capsicum* pods (Andrews, 1995; Bosland and Votava, 2012; Nuez et al., 2003). Curiously, most of these names have references to black pepper (Andrews, 1995).

The genus name was set in the early XVIIIth century by the physician-botanist Joseph Pitton de Tournefort (Tournefort, 1700). Unfortunately, the naming of the genus is also involved in mistery, since Tournefort did not leave an explanation of where the name *Capsicum* came from, although, two likely possibilities are considered as the most likely to be certain: one says that *Capsicum* comes from the Greek word *Kapso* meaning, "to bite" (pungent), whereas the other says it is from the Latin *Kapsakes* or *Kapsa* meaning

"pod". Both terms make reference to pepper pods most distinctive characteristics, its pungent taste and its shape, respectively (DeWitt and Bosland, 1996; Nuez et al., 2003).

To this day, the term *Capsicum* is usually reserved for taxonomic purposes while the previously mentioned terms are used as tradition dictates, despite the confusion it might cause (Bosland and Votava, 2012). Thus, in some cases, a specific variety can have different names depending on its origin, its maturity stage or even if it is consumed dry or fresh. This is especially true for Mexican varieties such as 'guajillo', which is also known as 'pulla' depending on the region, or the 'ancho' that could be called 'ancho mulato' when the ripe colour is dark brown instead of red. Alternatively, it can be called 'ancho Poblano' if it is from Puebla, Mexico. In addition, one of Peru's most extended varieties is named 'ají escabeche' (also known as 'amarillo' or 'verde') when consumed fresh but changes nomenclature to 'ají mirasol' when consumed dried (APEGA et al., 2009). Another interesting case where the terminology is ambiguous is with paprika. The term is used today to refer to the plant, to a variety, and to the powder obtained through the grinding of dried pepper fruits. This powder can have different grades of pungency, ranging from sweet to very pungent, and can be obtained from several varieties depending on where it is produced. Despite that, all of them get the designation of paprika, or 'pimentón' in Spanish (Bosland and Votava, 2012).

In Spain, the term 'pimiento' is used to refer to non-pungent types whereas 'guindilla' is reserved for pungent peppers, usually cayenne shaped (Nuez et al., 2003). Also, the Spanish terms *Pimiento Morrón*, *Morrón de Cascos* or *Pimiento de Morro* (*i.e.* resembling the nose of a cow, *Morro* in Spanish) may generate some confusion because they are used to refer to several pod types of sweet bell peppers (from blocky to rectangular shapes) with medium-large sized pods, A_1, A_2, A_3, B_1 and B_2 types from Pochard classification index, as well as their round/heart-shaped relatives called *Morrón de Bola* or *Morrón de Conserva*, *P* type (Pochard, 1966; Rodríguez-Burruezo et al., 2016).

3| Taxonomy of *Capsicum*

All pepper forms belong to the *Solanaceae*, a huge family that includes a wide range of important cultivated vegetables, such as tomato (*Solanum lycopersicum* L.), potato (*Solanum tuberosum* L.) and eggplant (*Solanum melongena* L.), along with other economically important plants such as tobacco (*Nicotiana tabacum* L.) or petunia (*Petunia* spp.). This complex of around 100 genera and more than 2.500 species is still poorly studied and little is known about the relationships among its species (Olmstead et al., 2008; Theodoro Hunziker, 2001).

Within the *Solanaceae* family, peppers are inserted in the *Solanoideae* sub-family, *Solaneae* tribe, *Capsinae* sub-tribe and in the *Capsicum* genus, a small group of species with a remarkable economic relevance all around the world (Theodoro Hunziker, 2001). Taxonomically, *Capsicum* is extremely complicated to classify due to the wide range of forms presented within the genus. This is not only true among species, but also among specimens as well.

Since the introduction of the first taxonomic classification, up until the early XXth century, the list of species included in this taxon has been in constant change, ranging from a single species up to several dozens. World's first botanists to work with the *Capsicum* genus had limited access to germplasm collections and, therefore, most classifications were based on morphological traits of a single or maybe a few individuals from herbarium collections. This poor representation of individuals lead to incorrect inclusion of non-*Capsicum* species, to classification of duplicates as distinct species and to wrong exclusion of some species from the genus (Andrews, 1995; Bosland and Votava, 2012; Nuez et al., 2003).

Capsicum taxonomy arrived at a turning point, in the mid XXth century, when Armando T. Hunziker, Charles B. Heiser and Paul G. Smith published several works that transformed *Capsicum* taxonomy. Thus, the former proposed the division of the genus in three sections: *Tubocapsicum*, *Pseudoacnistus* and *Capsicum*, the first two being monospecific and the latter a pluriespecific section containing all the pepper forms (Hunziker, 1956). Furthermore, morphological descriptors, especially flower traits, were essential to *Capsicum* classification and, until that moment, the genus included only rotated or sub-rotated corollas. In 1958, Heiser and Paul G. Smith described *C. cardenasii*, named after its collector Martín Cárdenas, as the first *Capsicum* species with campanulated corollas (Heiser and Smith, 1958). Then, a few years later, Hunziker described *C. scolnikianum*, also with campanulated corollas, corroborating Heiser and Smith's findings (Hunziker, 1961).

By this time, new methods were introduced, improving by many-fold the precision of taxonomic classifications. Those methodologies remained to this day and are now

standard to any species classification work (Russo, 2012). Hence, numerical taxonomy (Pickersgill et al., 1979), archaeological data (Kraft et al., 2014; Pickersgill, 1969), crosspollination studies (Eshbaugh, 1975; Heiser and Smith, 1958; Onus and Pickersgill, 2004; Pickersgill, 1971; Smith and Heiser, 1957), bioactive compounds analysis (Ballard et al., 1970; Jensen et al., 1979; McLeod et al., 1979a; Rodríguez-Burruezo et al., 2010; Zewdie and Bosland, 2000), cytological and karyotype studies (Moscone et al., 2007; Pickersgill, 1977; Pozzobon et al., 2006), and molecular markers (Baral and Bosland, 2004; Ibiza et al., 2012; Ince et al., 2010; Votava et al., 2002; Walsh and Hoot, 2001) are examples of methodologies present in the published reports around and after that period. In addition, another important breakthrough in the *Capsicum* taxonomy field was Barbara Pickersgill's innovative work, which proved that wild species were vital to understand the complexity of the genus and that they were related to the cultivated species. A series of posterior studies confirmed her findings (Heiser et al., 1971; McLeod et al., 1983; Pickersgill, 1971).

Recognizing the difficulty of *Capsicum* taxonomy, the International Board of Plant Genetic Resources (IBPGR) of the Food and Agriculture Organization (FAO) of the United Nations (UN) elaborated a plan of action, in 1983, in order to standardize terminology, to develop key descriptors and to give advice on sample collection for *Capsicum* specimens (IBPGR, 1983). From this plan of action resulted the standardized *Capsicum* descriptors that are still used today for characterization of germplasm of that genus (Bioversity International, 2019; IPGRI, 1995).

The mentioned efforts significantly contributed to enlighten *Capsicum* taxonomy and enabled to establish a list of species that remained somewhat stable till this day. Notwithstanding, it is likely that the number of recognized species change in the next future, because extensive exploratory expeditions to diversity centres allied to phylogeny studies may provide us with specimens still unknown to humankind and possibly new unknown relationships among them (Barboza, 2011; Barboza et al., 2019; Barboza and Bianchetti, 2005; Nee et al., 2006). Thus, the latest works consider as many as 43 species within the *Capsicum* genus (Barboza et al., 2019; Carrizo García et al., 2016, 2013; Moscone et al., 2007; Pozzobon et al., 2006; Russo, 2012; Silvar and García-González, 2016) (Table 3).

Table 3 - List of currently recognized species within the *Capsicum* genus arranged alphabetically. Adapted from Moscone et al. (2007) and Barboza et al. (2019).

Section	Species	Author
Tubocapsicum	*Capsicum anomalum*	Franchet & Savatier
Pseudoacnistus	*Capsicum brevifolium*	(Sendtn.) Hunz.
Capsicum	*Capsicum annuum var. annuum**	Linnaeus
	*Capsicum annuum var. glabriusculum**	(Dunal) Heiser & Pickersgill
	*Capsicum baccatum var. baccatum**	Linnaeus
	Capsicum baccatum var. pendulum	(Willd.) Eshbaugh
	Capsicum baccatum var. umbilicatum	(Vellozo) Hunz. & Barboza
	Capsicum benoistii	Hunz. ex Barboza
	Capsicum buforum	Hunz.
	Capsicum caatingae	Barboza & Agra
	Capsicum caballeroi	M. Nee
	Capsicum campylopodium	Sendtn.
	Capsicum cardenasii	Heiser & Smith
	Capsicum ceratocalyx	M. Nee
	Capsicum chacoense	Hunz.
	*Capsicum chinense**	Jacq.
	Capsicum coccineum	(Rusby) Hunz.
	Capsicum cornutum	(Hiern.) Hunz.
	Capsicum dimorphum	(Miers) Kuntze
	Capsicum eshbaughii	Barboza
	Capsicum eximium	Hunz.
	Capsicum flexuosum	Sendtn.
	Capsicum friburgense	Bianchetti & Barboza
	Capsicum frutescens	Linnaeus
	Capsicum galapagoense	Hunz.
	Capsicum geminifolium	(Dammer) Hunz.
	Capsicum hookerianum	(Miers) Kuntze
	Capsicum hunzikerianum	Barboza & Bianchetti
	Capsicum lanceolatum	(Greenm.) Morton & Standley
	Capsicum longidentatum	Agra & Barboza
	Capsicum longifolium	Barboza & Leiva
	Capsicum minutiflorum	(Rusby) Hunz.
	Capsicum mirabile	Mart
	Capsicum neei	Barboza & X. Reyes
	Capsicum parvifolium	Sendtn.
	Capsicum pereirae	Barboza & Bianchetti
	Capsicum piuranum	Barboza & Leiva
	Capsicum praetermissum	Heiser & Smith
	Capsicum pubescens	Ruiz & Pav.
	Capsicum recurvatum	Witas.
	*Capsicum rhomboideum**	(Dunal) Kuntze
	Capsicum schottianum	Sendtn.
	Capsicum scolnikianum	Hunz.
	Capsicum tovarii	Eshbaugh, Smith & Nickrent
	Capsicum villosum	Sendtn.

* Indicates that a synonymous name can still be found in the literature: *Capsicum annuum* var. *annuum* = *Capsicum cordiforme* (Mill.); *Capsicum annuum* var. *glabriusculum* = *Capsicum annuum* var. *minus* (Figherhuth), *Capsicum annuum* var. *baccatum* (Terpó), and *Capsicum annuum var. minimum* (Mill.) Heiser, *Capsicum annuum var. aviculare* (D'Arcy and Eshbaugh); *Capsicum baccatum* var. *baccatum* = *Capsicum angulosum* (Mill.), *Capsicum microcarpum* (Cav.); *Capsicum chinense* = *Capsicum sinense* (Murr.); *Capsicum rhomboideum* = *Capsicum ciliatum* (Kunth) Kuntze.

4| Origin, domestication and diffusion of *Capsicum*

4.1 The origin of *Capsicum*

Evidence suggests that *Capsicum* originated in South America, more precisely in Bolivia (McLeod et al., 1982; Moscone et al., 2007; Pickersgill, 1969). Although there is still discussion regarding some aspects, there is consensus among researchers on McLeod's et al. (1982) hypothesis, which proposes that *Capsicum* ancestor originated in the semi-arid highlands of south-central Bolivia in a designated "nuclear area" delimited by the cities Aiquile (to the west), Valle Grande (southeast) and Comarapa (northeast) (McLeod et al., 1982).

There is also consensus on *Capsicum* monophyly (Carrizo García et al., 2016; Olmstead et al., 2008; Walsh and Hoot, 2001). Hence, authors propose *C. chacoense*, or an ancestor-like, to be the most likely genus ancestor, based on karyological, cytological and molecular data (Jensen et al., 1979; Moscone et al., 2007). Thus, theory suggests that *C. chacoense* (or its ancestral form) gave rise to both white and purpled-flowered groups ancestors by migrating to Bolivian lowlands and to Andean highlands, respectively, and through mutation and natural selection gave place to new forms, which would be the ancestors of today's species (McLeod et al., 1982; Moscone et al., 2007). As a result, a plethora of forms arose, probably dispersed by birds and water courses, long before the first humans populated those regions (Bosland and Votava, 2012; DeWitt and Bosland, 1996; McLeod et al., 1982; Pickersgill, 1984).

Eventually, the purple-flowered ancestor, possibly *C. eximium*, through genetic drift, selection and adaptation, gave origin to *C. cardenasii*. Later, this species would give place to domesticated form *C. pubescens* (McLeod et al., 1982, 1979b). Furthermore, white-flowered group ancestor followed two separate speciation processes that culminated in the bearing of several different species. Thus, individuals that migrated to the Bolivian dry lowlands originated *C. baccatum* var. *baccatum* that eventually, through domestication, would give origin to cultivated form *C. baccatum* var. *pendulum* and *C. baccatum* var. *umbilicatum* (Scaldaferro et al., 2018). Then, that same ancestor got out of the Bolivian "nuclear area" via the Mizque River and, eventually, reached Amazon basin humid regions where through selection and adaptation processes originated the ancestor of the *C. annuum* complex (McLeod et al., 1982).

Capsicum annuum var. *glabriusculum* (Dunal) Heiser & Pickersgill, which still can be found as wild and semi-wild forms, is the proposed ancestor of *C. annuum* complex (Aguilar-Meléndez et al., 2009; Hayano-Kanashiro et al., 2016; Heiser and Pickersgill, 1975; Moscone et al., 2007). This species name changed several times and in some literature it can still be read as *C. annuum* var. *minus* (Figherhuth), *C. annuum* var. *baccatum* (Terpó), *C. annuum* var. *minimum* (Heiser and Pickergill) or *C. annuum* var.

aviculare (D'Arcy and Eshbaugh), although, today, the accepted term is *C. annuum* var. *glabriusculum* (Barboza, 2011; Hayano-Kanashiro et al., 2016; Heiser and Pickersgill, 1975). Hence, like for *C. chacoense*, a two-way evolutionary line gave birth to three distinct forms of the genus. On one hand, the migration of *C. annuum var. glabriusculum* to the north part of South America, and eventually North and Central America, especially Mexico, gave place to *C. annuum* var. *annuum* (McLeod et al., 1982; Moscone et al., 2007). And on the other, movements of *C. annuum* var. *glabriusculum* towards the Amazonic basin gave origin to the other *annuum* complex species, *C. chinense* and *C. frutescens*, completing the group of cultivated species of the *Capsicum* genus (McLeod et al., 1982; Moscone et al., 2007).

Hence, white-flowered species *C. annuum, C. chinense* and *C. frutescens* form the *annuum* complex and can produce viable hybrids among them (Baral and Bosland, 2004; Walsh and Hoot, 2001). Furthermore, the *annuum* complex is a completely separate taxon from the other white-flowered complex, *C. baccatum*, as well as from the purpled-flowered complex, *C. pubescens*, and the obtention of viable hybrids among these complexes is limited by strong incompatibility barriers only overcome using hybridization approaches (Manzur et al., 2015; Yoon et al., 2006; Zijlstra et al., 1991). The relationships among these species have been extensively studied throughout the years and is, in fact, one of the most important pieces of evidence to validate the evolutionary line of the *Capsicum* genus and the origin of the domesticated forms (Eshbaugh, 1983; Moscone et al., 2007).

4.2 Domestication and diffusion of *Capsicum*

Since its break out from the "nuclear area", *Capsicum* ancestors went through several migration and speciation processes, giving place to several species distributed through a wide range of environments along South, Central and North America (Hernández-Verdugo et al., 1999; Moscone et al., 2007). Those species underwent independent domestication processes resulting in increase of genetic distances and creation of a plethora of different forms, long before first human contact (Bosland and Votava, 2012; McLeod et al., 1982; Pickersgill, 1984). Eventually, domestication enabled plant modification, as well as the breeding of new forms, colours, sizes, grades of pungency, and even non-pungent varieties, completely different forms from the small wild types humans first encountered (DeWitt and Bosland, 1996; Heiser et al., 1971).

Regarding that, based on several archaeobotanical records, researchers estimate that pepper was first domesticated around 7000 years ago. The oldest macroremains ever recovered were found in Puebla and Tamaulipas, in Mexico (Kraft, 2009; Kraft et al., 2014; Russo, 2012). Hence, evidence suggests that *C. annuum* was domesticated in Mexico, *C. chinense* in the northern lowlands of Amazonia, *C. frutescens* in the

Caribbean, *C. baccatum* in lowlands of central Bolivia, and finally, *C. pubescens* in the mid-elevation lands of Southern Andes (Aguilar-Meléndez et al., 2009; Carrizo García et al., 2016; Eshbaugh, 1993; Heiser et al., 1971; Kraft et al., 2014; Loaiza-Figueroa et al., 1989; Moscone et al., 2007; Pickersgill, 1984, 1971, 1969; Scaldaferro et al., 2018).

Capsicum fruits had huge relevance to the first human tribes in the Americas, both as a spice and as medicine, as several recovered archaeological data can accredit for (Andrews, 1995; Nuez et al., 2003; Russo, 2012). For example, the Aztecs had a diet composed by corn and bean spiced up with tomato, pepper and cacao. Likewise, the Mayans, Incas and Amazonian tribes, frequently included *Capsicum* pungent fruits, as a spice or as sauce, in their diets (Andrews, 1995; Hernández-Verdugo et al., 1999; Nuez et al., 2003). Contrastingly, in the XVth century Europe, oriental spices were the main food preservative and seasoning ingredient, particularly the black pepper (*Piper nigrum* L.). In that era, spices had remarkable relevance in the world's politics, and who controlled spices trade routes, controlled the world (Andrews, 1995; Nuez et al., 2003). Hence, Columbus set sail, aiming at finding a shorter route than the Portuguese to the Orient. Instead, Columbus and Spanish explorers found a new continent and a completely different variety of plants and foods, among them peppers, a vital element in the Mesoamerican culture, as Columbus diary entries from around that era emphasize «*… there is a great amount of cotton, thin and long… and there is also ají, their black pepper, that is worth more than black pepper, and nobody eats without it, …*» (Andrews, 1995; Nuez et al., 2003). After its introduction in Spain, as a cheaper alternative to Asian black pepper, pepper rapidly spread across the Old World. First, mainly *C. annuum* specimens, from Spain to Italy, France, England and Central Europe. Secondly, *C. chinense* varieties, exported by the Portuguese explorers from Brazil to Eastern Europe, Africa and Asia, via Portuguese colonies in those continents (Andrews, 1995; Eshbaugh, 1983; Nuez et al., 2003). By mid XVIth century, countries like Germany, Italy and England had extensively cultivated pepper throughout the country. Finally, Spanish explorers introduced pepper in the United States by the end of XVIth century, where is now a relevant element of the country's agriculture and gastronomy (Bosland and Votava, 2012; DeWitt and Bosland, 2009; Hernández-Verdugo et al., 1999). After a rapid global diffusion based on its use as a spice, diversification brought new uses and even non-pungent varieties, that nowadays prevail over the pungent ones in the occidental cuisine. Likewise, *Capsicum* pods had a tremendous impact and forever changed Mediterranean, Chinese and Indian gastronomy (Andrews, 1995; Bosland and Votava, 2012; Nuez et al., 2003).

5| *Capsicum* germplasm with emphasis on Spanish landraces

5.1 Spain, centre of diversity of *Capsicum annuum*

Among the cultivated species, *C. annuum* is the most diverse, economically relevant and studied species worldwide, and includes both non-pungent and pungent types (Bosland and Votava, 2012; DeWitt and Bosland, 1996; Nuez et al., 2003). During the Discovery Era, Spain was the main entrance for all the materials brought from Mexico, Caribbean and South America (Andrews, 1995; Nuez et al., 2003). Furthermore, due to the introduction of such amount of germplasm, mainly *C. annuum* varieties, a plethora of Spanish ecotypes and landraces, adapted to a wide range of agroclimatic conditions, were originated, selected and perpetuated until our days by generations of farmers (Nuez et al., 2003) (Table 4). As a result, Spain is nowadays Europe's biggest and the world's sixth biggest pepper producer (FAO, 2019; MAPA, 2019).

Fruit of more than five-hundred years of selection performed by generations of farmers, *C. annuum* fleshy, big-fruited bell-peppers, called *Morrón* and its derivates, represent today the largest number of EU Protected Designations of Origin (PDO) and Protected Geographical Indications (PGI) in Spain, and make part of the country's agriculture and gastronomic heritage (MAPA, 2019; Nuez et al., 2003; Rodríguez-Burruezo et al., 2016) (Table 4). Despite such ethnobotanic heritage, Spanish pepper production is based, for the most part, on F_1 hybrids from 'California Wonder', 'Lamuyo' and 'Dulce Italiano' types, which have displaced traditional and ancient materials, increasing their risk of disappearing (Hammer, 2004; Hammer et al., 2003; Lanteri et al., 2003; Votava et al., 2005). In fact, the abandonment of ancient materials threatens agrodiversity and has only been mitigated by the efforts of institutions through germplasm collecting expeditions for the past half century (Alonso et al., 2018; DeWitt and Bosland, 1996; Hammer, 2004; Rodríguez-Burruezo et al., 2016).

Fortunately, a growing interest for the "taste of the past" by consumers, the challenge of adapting to climate change and the pursue for sustainable production systems are contributing to the reintroduction of landraces (Brugarolas et al., 2009; Casals et al., 2011; Egea-Fernández et al., 2018; Hurtado et al., 2013; Rivera et al., 2016). In summary, the natural ethnobotanic heritage throughout the Spanish territory provides a remarkable amount of genetic diversity for pepper breeding programs and consequently for food security (Casals et al., 2011; Egea-Fernández et al., 2018; Rodríguez-Burruezo et al., 2016).

On that matter, Spain encompasses a plethora of landraces and ecotypes throughout its territory, where the majority are fleshy, big-fruited peppers (> 50 g) with a wide range of varietal types and fruit shapes, also known as *Pimiento Morrón* and *Morrón de Conserva*. In addition to those, in smaller number, we can also find relevant small-sized

fruit varieties, characterized by thin flesh and pungent taste, which are common to the insular territories of Canary Islands (Nuez et al., 2003; Rodríguez-Burruezo et al., 2016).

Hence, within the *Morrón* types, with three or four lobules, the most relevant landraces are 'Valenciano', 'Trompa/Morro de Vaca de Murcia', 'Pimiento de Infantes', 'Pimiento de Litro', 'Morrón de Fresno de la Vega y Benavente' and 'Largo de Reus' (Pochard A_1, A_2, A_3, B_1 and B_2). Regarding *Morrón de Conserva* (*P* type), the most important varieties are 'Tudelano' from Navarra, and 'Calahorra' and 'Morrón de Luesia' from Aragon. In addition, closely related to both varietal types mentioned above, we may find another kind of large thick-fleshed peppers with triangular or conical shape, such as 'Pimiento Asado del Bierzo' (B_3), 'Ros Mallorquí' and 'Najerano' (C_3), as well as 'Pimiento del Piquillo de Lodosa' and 'Pico de Mendavia' (C_4) (Pochard, 1966; Rodríguez-Burruezo et al., 2016) (Table 4).

Regarding medium-small sized peppers (B_3, B_4 and C_4) we can find several important ones, particularly in the North of Spain, such as Galician DOP and PGI ecotypes, collected immature and before reaching its full size, like 'Arnoia' or 'Oímbra' from Orense, 'Pemento de Herbón' and 'Pemento do Couto' from A Coruña and Pontevedra, or the 'Pemento do Mougán', cultivated in Lugo. These materials are believed to descend from a genetic influx from Mexico in the XVIIth century by the Franciscans (Rodríguez-Bao et al., 2004). In addition, within B_4 peppers we can find 'Pimienta Palmera', used to make Canary Islands dip *Mojo palmero*. Still within medium-small peppers, we can find a variety called 'Gernikako Piperra' from the Basque Country, with an elongated and pointy shape (C_2), that is consumed fried (immature) or dried (mature), also known as 'choricero', which is related to the French IGP 'Espellete' (Pochard, 1966; Rivera et al., 2016; Rodríguez-Burruezo et al., 2016) (Table 4).

Another important group of traditional varieties within the Spanish agriculture are those of elongated shape (C_1), medium or large sized, presenting or lacking shoulders, which can be called *Cornicabra* or *Guindillas*. Thus, for the first, fruits are harvested red and dried in order to make *Pimentón de la Vera*, in the regions of Murcia, Andalusia, Castilla-La Mancha, and, especially, in Extremadura, being the most relevant ecotypes 'Ocal' or 'Agridulce' and their derivates 'Jaranda' also known as 'Jariza' (Nuez et al., 2003; Rodríguez-Burruezo et al., 2016). Finally, *Guindillas* present a wide range of sizes within the elongated shape group, depending on the variety, although they are usually thinner than the ones included in *Cornicabra* group. As recognized varieties we may find 'Guindilla de Ibarra' or 'Alegrías Riojanas' which are consumed pickled (immature) or roasted and canned (mature), respectively (Nuez et al., 2003; Pochard, 1966; Rodríguez-Burruezo et al., 2016) (Table 4).

Finally, an important group of small and round fruits (*N* type) is consumed almost entirely as food colouring agent, known as 'Ñora' or 'Pimiento de Bola'. Within this particular group we may encounter several varieties throughout many Spanish regions,

however, the most relevant one is *Pimentón de Murcia* for which there is a mutant selection called 'Negral' which carries the *cl* allele, responsible for the characteristic brown colour by blocking the chlorophyll degradation during the maturation of the fruit (Nuez et al., 2003; Pochard, 1966; Rodríguez-Burruezo et al., 2016; Rodríguez-Burruezo and Nuez, 2006) (Table 4).

Table 4 – Spanish pepper PDOs and PGIs and corresponding designation, registration year and region of production (MAPA, 2019; Nuez et al., 2003).

Variety	Designation (UE registration year)	Region of production
Arnoia	PGI Pemento da Arnoia (2010)	Orense
Bierzo	PGI Pimiento Asado del Bierzo (2006)	León
Bola	PDO Pimentón de Murcia (2001)	Murcia
Couto	PGI Pemento do Couto (2010)	A Coruña
Gernika	PGI Gernikako Piperra (2010)	Euskadi (Basque Country)
Herbón (Padrón)	PDO Pemento de Herbón (2010)	A Coruña and Pontevedra
Jaranda	PDO Pimentón de la Vera (2007)	Caceres
Morrón de Fresno	PGI Pimiento de Fresno y Benavente (2012)	León and Zamora
Mougán	PGI Pemento do Mougán (2014)	Lugo
Oímbra	PGI Pemento de Oímbra (2010)	Orense
Piquillo Lodosa	PDO Pimiento del Piquillo de Lodosa (1996)	Navarra
Riojano	PGI Pimiento Riojano (2004)	La Rioja

5.3 *Capsicum* germplasm collections

Spanish institutions detain a considerable number of *Capsicum* accessions, encompassing country's most relevant varieties, like the ones mentioned in the previous chapter, some obscure or obsolete materials, foreign and wild varieties, and even germplasm from *Capsicum* related species (Table 5) (Alonso et al., 2018; Díez et al., 2018).

Within these collections, two levels can be perceived. On one hand, active collections, usually managed by breeders, encompass a small number of accessions and varietal types, representative of the existent diversity for a crop or region, which in many cases provided pre-breeding materials towards the development of relevant PDOs and PGIs (Díez et al., 2018; Rodríguez-Burruezo et al., 2016). On this matter, the most impressive collections are detained by the Instituto Vasco de Investigación y Desarrollo Tecnológico (NEIKER), the Instituto Navarro de Tecnologías e Investigación Agroalimentaria (INTIA), the Centro de Investigaciones Agrarias de Mabegondo (CIAM), the Centro de Investigaciones Científicas y Tecnológicas de Extremadura (CICYTEX-La Orden), and the Instituto Murciano de Investigación y desarrollo Agroalimentario (IMIDA) (Facundo et al., 2014; NEIKER-Tecnalia, 2016; Nuez et al., 2003; Rodríguez-Bao et al., 2004; Taboada Arias et al., 2007).

And on the other, at a much larger scale, some collections are devoted to collection, documentation, regeneration and distribution of the diversity inherent to one or several species from one or several crops (Díez et al., 2018). These institutions are responsible

for the highest amount of genetic diversity by conserving large collections of landraces, ecotypes, modern varieties, pre-breeding materials, semi and wild relatives, obsolete materials, endangered species, secondary and tertiary genepool materials, including national and foreign materials (Alonso et al., 2018; Díez et al., 2018). Regarding *Capsicum* resources within Spain, the Banco de Germoplasma del Instituto de Conservación y Mejora de la Agrodiversidad Valenciana (COMAV), the Banco de Germoplasma de Hortícolas del Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA) and the Centro de Recursos Fitogenéticos del INIA (CRF-INIA) are the most relevant institutions, encompassing almost 2000 catalogued pepper accessions (Alonso et al., 2018; Díez et al., 2018) (Table 5).

Genebanks were created with the goal of preserving allelic or gene combinations in order to mitigate diversity loss caused by the introduction and wide adoption of modern cultivars. Hence, seeds from distinct materials were conserved for future generations to exploit as a source of advantageous traits in breeding programs (Díez et al., 2018). In Spain, such collections started to be constructed around 1980, when Joaquín Costa (IMIDA), Ramiro Gil (CITA), Fernando Nuez (COMAV), and respective research groups, initiated exhaustive collection expeditions in order to collect and catalogue Spanish indigenous germplasm to be used in future breeding programs and to promote traditional agriculture (Carravedo et al., 2005; Díez et al., 2018, 1998; Rodríguez-Burruezo and Nuez, 2012). Today, these institutions account for a remarkable number of pepper accessions from all parts of the globe. Based on the latest data, combined, CRF-INIA, COMAV and CITA conserve 1960 *Capsicum spp*. accessions, which 93.93% (1841 acc.) correspond to *C. annuum* accessions and 88.57% (1736 acc.) correspond to Spanish landraces from all Spanish regions (CRF-INIA, 2019) (Table 5). Furthermore, the COMAV's genebank accounts for 854 accessions, 76.63% (680 acc.) of which correspond to accessions from all Spanish territories, whereas CITA encompasses 966 entries, from which 96.58% (933 acc.) correspond to Spanish materials. The most represented region of Spain is Andalusia with an 18.78% (326 acc.) of all Spanish materials conserved at CRF-INIA (CRF-INIA, 2019; Rodríguez-Burruezo et al., 2016) (Table 5).

On a global scale, it is worth mentioning other remarkably important germplasm banks such as The Centre for Genetic Resources (CGN) from the Netherlands, the United States Department of Agriculture Genebank (USDA) and the World Vegetable Centre (AVRDC) from Taiwan (AVRDC, 2019; CGN, 2019; USDA et al., 2019). These institutions detain huge collections encompassing materials from all around the world, including Spanish landraces and ecotypes, and represent an excellent source of variation for breeding programs. For instance, CGN has around 1141 *Capsicum* spp. accessions, USDA has 6301 and the AVRDC genebank has an impressive 8375 *Capsicum* spp. accessions (AVRDC, 2019; CGN, 2019; USDA et al., 2019).

Table 5 – Number of *Capsicum* spp. accessions conserved at CRF-INIA, COMAV and CITA and their distribution based on number of *Capsicum annuum* accessions and region of origin (CRF-INIA, 2019).

Category	CRF-INIA	COMAV	CITA
Total number of accessions	1960	854	966
Number of *Capsicum annuum* accessions	1841	763	961
Andalusia	326	163	163
Aragon	62	7	55
Balearic Islands	83	2	51
Basque Country	68	3	65
Canary Islands	191	88	31
Cantabria	38	6	32
Castilla-La Mancha	152	76	75
Castile and León	152	18	134
Catalonia	68	61	7
Community of Madrid	17	2	7
Community of Navarra	28	1	26
Extremadura	192	52	140
Galicia	71	2	68
La Rioja	42	9	32
Principality of Asturias	34	16	18
Region of Murcia	57	36	15
Valencian Community	155	138	14
Total number of Spanish accessions	1736	680	933

5.4 Landraces as source of variation

Landraces and ancient materials represent an important source of variation as opposed to modern varieties (Casals et al., 2011; Fita et al., 2015; Hammer and Khoshbakht, 2005; Lanteri et al., 2003; Rivera et al., 2016). Furthermore, *Capsicum* spp., and especially *C. annuum*, encompass a wide genetic diversity, which is of paramount importance for detection and exploitation of advantageous allelic combinations (Jing et al., 2016; Mundt, 2014; Rivera et al., 2016; Silvar and García-González, 2017; Zonneveld et al., 2015). Therefore, the considerable number of landraces and ecotypes distributed throughout the Spanish territory represents an important resource for breeding programs and for the development of more resilient varieties to several abiotic stresses, as well as for improved nutritional content varieties (Casals et al., 2011; Egea-Fernández et al., 2018; Figàs et al., 2018a; Fita et al., 2015; Parisi et al., 2017; Prohens et al., 2017).

Traditional varieties are a result of a long evolutive and selective process. In the case of Spanish landraces, the heterogeneity of climatic conditions, soils and horticultural practices throughout the country's landscape created dozens of different varieties adapted to local agroclimatic conditions. As a result, these individuals are much more resilient to climate change and more prone to prevail under low input conditions, as well

as water, salt and heat stress conditions (Fita et al., 2015; Muñoz-Falcón et al., 2008; Parisi et al., 2017; Rivera et al., 2016).

Furthermore, peppers are naturally rich in several nutritional compounds such as carotenoids, phenolic compounds, fats, oils, and minerals, making them one of the most relevant foods in the world (DeWitt and Bosland, 2009; Morales-Soto et al., 2014; Pellegrini et al., 2003; Pérez-López et al., 2007b; Ribes-Moya et al., 2018; Rodríguez-Burruezo et al., 2010). Thus, traditional varieties could represent an important source of variation for improved fruit content due to the their inherent rich genetic variability (Egea-Fernández et al., 2018; Figàs et al., 2018a; Parisi et al., 2017; Ribes-Moya et al., 2018; Rodríguez-Burruezo et al., 2005). In addition, the fact that fruits are consumed in two maturity stages makes them even more interesting, since those which are eaten immature (green) can present interesting internal content values if consumed fully ripen, and vice-versa (Ribes-Moya et al., 2018). Thus, it is of paramount importance to evaluate landrace varieties for both maturity stages in order to identify those with the best internal characteristics to be consumed in a determinate maturity state or to be used to improve other varieties (Rodríguez-Burruezo and Nuez, 2006). Traditional varieties are excellent candidates to search for new sources of variation regarding internal content and should be exhaustively characterized for their bioactive compounds content such as ascorbic acid, phenols, carotenoids, capsaicinoids, minerals, etc., as well as for their possible industrial and medicinal applications (Moreno-Peris et al., 2012; Rodríguez-Burruezo et al., 2010, 2009).

6| *Capsicum* breeding

6.1 Resistance to biotic and abiotic stresses

Plants, contrarily to animals, lack the ability to move in order to escape adverse conditions or the menace of a predator. Thus, plants developed mechanisms which enable their survival under difficult conditions (Chhapekar et al., 2018). Regarding that, peppers, like most crops, are susceptible to a plethora of pests, diseases and physiological disorders and, therefore, breeding towards highly resistant varieties has always been a major goal in *Capsicum* breeding programs (Khush, 2002; Silvar and García-González, 2017). Thankfully, the *Capsicum* genus encompasses a wide genetic diversity for breeders to take advantage of and search for new advantageous allelic combinations (Mundt, 2014; Silvar and García-González, 2017). Despite detecting sources of resistance being an incredibly laborious process, during the last decades, remarkable progress has been made regarding pepper breeding for resistance to both biotic and abiotic stresses (Russo, 2012; Vats, 2018).

In terms of biotic resistance, several resistance genes or QTLs against bacterial, fungal, viral, and nematode diseases have been described over the last few decades. Some examples are *Xanthomonas campestris* (Jones et al., 2002; Pierre et al., 2000), *Phytopthora capsici* (Candole et al., 2010; Kim et al., 2008), *Leveillula taurica* (Lefebvre et al., 2003), Cucumber mosaic virus (Caranta et al., 1997; Chaim et al., 2001), Tomato spotted wilt virus (Moury et al., 2000), or *Meloidogyne incognita* (Djian-Caporalino et al., 2001; Fazari et al., 2012), among others (Chhapekar et al., 2018; Kim et al., 2017; Russo, 2012; Wang and Bosland, 2006). Notwithstanding, pathogens are in constant evolution and many overcome resistances within a few generations. The high incidence rate of a specific pathogen and widespread monocultivation of varieties sharing the same R resistance genes favours the overcome of resistance (Djian-Caporalino et al., 2014; Pilet-Nayel et al., 2017; Russo, 2012). In addition, for the most part, resistance to pathogens have been based on effects of R genes or major QTLs, to which resistance is more likely to be overcome (Djian-Caporalino et al., 2014; Mundt, 2014; Pilet-Nayel et al., 2017). The pyramiding of several genes or QTLs of minor and major additive effect has been reported as a more effective and durable mechanism (Djian-Caporalino et al., 2014; Mundt, 2014; Pilet-Nayel et al., 2017). However, the identification of QTLs, especially those of minor effect and epistatic, has been limited by researchers knowledge and available technology (Pilet-Nayel et al., 2017; Russo, 2012).

The labour inherent to the discovery and characterization of resistance to abiotic stresses is, in general, more complex than for biotic stresses, due to the polygenic nature of its mechanisms. Despite that, major contributions have been reported in the last years for

several abiotic disorders in *Capsicum*, findings of paramount importance as we prepare for a world under the effects of climate change (Raza et al., 2019). Hence, the development of varieties with the ability to withstand such difficult environments are vital to avoid loss of production. Over the past years, several remarkable works have shed light into the mechanisms controlling resistance to several abiotic stresses, such as heat (Guo et al., 2016, 2014; Li et al., 2015), drought (Hong and Kim, 2005; Huang et al., 2019; Sahitya et al., 2019; Shivakumara et al., 2017), cold (Hwang et al., 2005; Li et al., 2016), and salt (Bojórquez-Quintal et al., 2014; Jing et al., 2016). Furthermore, collaboration between COMAV and Instituto Valenciano de Investigaciones Agrarias (IVIA) yielded relevant information regarding salt and water stress by identifying pepper varieties capable of sustaining such adverse conditions, which are already being tested as rootstocks (Penella et al., 2016, 2014, 2013). These results represent a major breakthrough, particularly for the Mediterranean conditions where pepper is massively cultivated and has an acute sensibility to these conditions, which will probably get worse due to climate change (Fita et al., 2015; Raza et al., 2019).

Despite of those advances, we are still far from fully understand the mechanisms involved in the response to abiotic stress and, therefore, far from being able to introgress those traits into new cultivars (Russo, 2012). In an era of low-cost high-throughput genotyping and unprecedented germplasm collections available (Díez et al., 2018; Elshire et al., 2011; He et al., 2014), an exhaustive characterization of synthetic populations such as MAGIC (Multiparental Advanced Generation Intercross), which favour recombination and allows for a wider genetic diversity, as they include more cycles of informative meiosis (Bandillo et al., 2013; Huang et al., 2015; Pascual et al., 2015), would enable a better dissection of genomic regions involved in the response against biotic and abiotic stress (Chhapekar et al., 2018; Russo, 2012). In fact, European Horizon 2020 funded projects, such as G2P-SOL (Linking genetic resources, genomes and phenotypes of Solanaceous crops), are already doing massive germplasm characterization and genotyping in order to catalogue existing resources as an important first step towards the exploitation of said resources (Alonso et al., 2018; "The G2P-SOL project," 2019).

6.2 Adaptation to low input conditions: the case of phosphorus

The period between the 1960's and the late 1990's is known as *The Green Revolution*, an era where technological advances enabled unprecedented crop yields and avoided a catastrophic food shortage in the developing nations of then (Khush, 2002). During those years, collaboration among researchers, such as Norman Borlaug (1914-2009), governmental agencies, the International Wheat and Maize Improvement Centre (CIMMYT), the International Rice Research Institute (IRRI), and local farmers,

combined with a concise strategy, was vital to the success of *The Green Revolution* (Khush, 2002). First, the introduction of wheat and rice dwarf varieties, carrying several resistance genes and adapted to a wide range of latitudes and environments resulted in significant yield increase. Secondly, governmental policies and substantial investment in infrastructure was vital in order to make ends meet. However, the most decisive factor was probably the use of high quality chemical fertilizers, particularly nitrate and phosphate, otherwise it would be difficult to achieve such levels of productivity (Khush, 2002; Lynch, 2007; Tilman et al., 2002). As a result, the use of chemical fertilizers became standard in modern agriculture, particularly in the developed countries, which were able to afford the associated costs (Mogollón et al., 2018).

Thus, phosphorus (P) is a non-organic mineral of major relevance within several physiochemical processes of plants (Schnug and Haneklaus, 2016a). Therefore, the depravation of P can have a severe impact on plant development (Lynch, 2007). In fact, P is the most limiting element for crop development in most of agricultural soils (Jones, 2012; Schnug and Haneklaus, 2016b; Vance et al., 2003). Around 30% to 40% of the world's arable land does not have the soil properties to make it available for plants (Cordell et al., 2009; Mogollón et al., 2018; Vance et al., 2003). Furthermore, P is usually uptaken by plants under the soluble forms $H_2PO_4^-$ or HPO_4^{2-} and the concentration of these orthophosphates is highly dependent on the soil solution pH and concentration of Al, Fe and Ca cations, with which forms strong complexes, making it unavailable for plants to assimilate (Jones, 2012; Schnug and Haneklaus, 2016b; Vance et al., 2003). Adding to that, the concentration of soluble P forms represents a very small fraction of total P in the soil solution (Schnug and Haneklaus, 2016b). In fact, the vast majority of P is strongly bonded to soil particles or is under the organic form, which needs to be mineralized and only then added to the soil solution (Schnug and Haneklaus, 2016b; Vance et al., 2003).

As a result, addition of P-enriched fertilizers is usually the adopted strategy to replenish P levels after each harvest (Cordell et al., 2009; Mogollón et al., 2018). However, this practice has several associated environmental and socio-economic problems. First, only a fraction of the added fertilizer is uptaken by crops, between 15% to 40% according to researchers, while the remaining ends up lixiviated or lost along with eroded soil (Lynch, 2007; Tilman et al., 2002; Vance et al., 2003). Plus, lixiviated fertilizer ends up contributing to eutrophication and hypoxia of water bodies, as reported for both Mediterranean and Baltic seas (Fernández and Selma, 1998; Kauranne and Kemppainen, 2016). Adding to that, P is a non-renewable resource obtained through mining, which has also several associated issues. One, P-mining has a severe impact on the environment due to its carbon, heavy metals and radio-nuclides emissions (Cordell et al., 2009; Schnug and Haneklaus, 2016b; Tilman et al., 2002). Two, rock-phosphate reserves are controlled by just three countries (China, Morocco and USA) and are being depleted fast. In fact, some authors estimate that these reserves could be completely

depleted by the end of this century (Cordell et al., 2009; Schnug and Haneklaus, 2016b; Vance et al., 2003). Third, P is becoming an extremely expensive resource, that is already unaffordable in many regions of the world, and its price will probably increase due to reduction of P reserves and increase of extraction and shipping costs (Cordell et al., 2009; Schnug and Haneklaus, 2016b; Vance et al., 2003). Finally, as reserves reach their lowest levels, rock-P quality also decreases (Cordell et al., 2009; Mogollón et al., 2018). Despite all that, demand for P-enriched fertilizers is believed to increase in the next decades, particularly in poor and developing countries (Mogollón et al., 2018; Schnug and Haneklaus, 2016a).

In the advent of climate change, some believe we are in need for a new *Green Revolution* that would enable us to increase food production without jeopardising the environment (Lynch, 2007; Mogollón et al., 2018; Raza et al., 2019). Hence, improving the ability of crops to uptake and efficiently use P is of paramount importance in order to reduce rock-P mining and fertilizer applications. Regarding that, the development of improved genotypes capable of yield under nutrient deficiency is conditioned by the researchers understanding of genetic mechanisms underlying the response. In the last years, several works have reported several genomic regions that are activated under said conditions for several crops (Hammond et al., 2009; Li et al., 2009; Lynch and Brown, 2001; Zhu et al., 2005). In addition, several authors have linked root architecture to enhanced mineral acquisition. Those studies indicate that root adaptations, such as increment of number of lateral roots, root hairs and cluster roots, change of root architecture to a topsoil foraging system, increment of organic acids production and phosphatases, root P transporters enhanced expression, and root cellular structure alteration, are found in a vast number of species and are correlated to a greater performance under low P conditions (Niu et al., 2013).

Peppers are one of the most important vegetables in the world, with an annual production close to 40 Mt and a dedicated cultivation area close to four Mha all around the world (FAO, 2019). Therefore, the development of varieties with enhanced P-uptake and P-use efficiency would significantly reduce the need for fertilizer applications. Consequently this would translate into cost reduction for farmers, a reduction of contaminants emission associated to rock-P mining, and finally a reduction of the amount of fertilizer that ends up lixiviating into lakes and rivers (Cordell et al., 2009; Tilman et al., 2002). Most importantly, the development of low P inputs adapted varieties would have a remarkable impact on yield of poor and low input agricultural systems of the undeveloped and developing world (Mogollón et al., 2018). Despite all that, and that there are several high-throughput methods meant to study the radicular system, the mechanisms controlling response and root morphological adaptations to low P conditions in pepper are still unravelled (Paez-Garcia et al., 2015; Pereira-Dias et al., 2015b).

Improving peppers ability to uptake and use P efficiently can be achieved if there is genetic variability from which to take advantage of. Regarding that, diversity related to those characters has been demonstrated for several crops (Bates and Lynch, 2001; Fernandez and Rubio, 2015; Fita et al., 2012, 2011; Lynch and Brown, 2001). Likewise, *Capsicum* spp., and particularly *C. annuum*, are remarkably diverse and are adapted to a wide range of environments and conditions and therefore tolerant to several abiotic stresses (Bosland and Votava, 2012; DeWitt and Bosland, 1996; Hwang et al., 2005; Jing et al., 2016; Sahitya et al., 2019). With that in mind, an exhaustive pepper germplasm screening should be incentivized in order to promote the discovery of candidate materials for improved root architecture and enhanced P uptake and use efficiency. Likewise, the ability to link phenotype to genotype is of paramount importance in order to introgress genomic regions controlling traits of interest into other materials (Prohens et al., 2017). Next Generation Sequencing (NGS) tools and Genome-Wide Association Studies (GWAS) have already demonstrated potential identifying genomic regions controlling traits of interest and providing thousands of molecular markers linked to said traits (Ahn et al., 2018; Celik et al., 2017; Guo et al., 2016; Nimmakayala et al., 2016a; Pascual et al., 2015). The use of high-throughput phenotyping and genotyping methods enables large scale characterization of collections and, therefore, may result in an accurate understanding of the mechanisms underlying the response to P depravation (Chhapekar et al., 2018; He et al., 2014; Paez-Garcia et al., 2015).

6.3 Improved fruit internal quality

Peppers are known to be one of the richest vegetables, providing a wide range of health-promoting compounds to almost all cultures around the world. Among those compounds we can find capsaicinoids, sugars, acids, vitamins, flavonoids, carotenoids, fats, oils, and minerals (DeWitt and Bosland, 2009; Morales-Soto et al., 2014; Pellegrini et al., 2003; Pérez-López et al., 2007b; Ribes-Moya et al., 2018; Rodríguez-Burruezo et al., 2010, 2009; Rodríguez-Burruezo and Nuez, 2006; Zonneveld et al., 2015). Thus, breeding for improved fruit quality is a major goal for pepper breeding programs. However, for a long period of time, fruit internal quality was relegated to a second plan while breeding programs focused, almost entirely, on variety's yield, resistance to biotic stresses, external quality, and longer shelf life, resulting in loss of sensorial quality (Casals et al., 2011; Egea-Fernández et al., 2018; Parisi et al., 2017).

As previously mentioned, interest for the "taste of the past" is contributing to stimulation of landraces cultivation due to the consumer's perception of these materials as a better source of nutrients and richer in flavour, as opposed to modern varieties (Brugarolas et al., 2009; Casals et al., 2011; Egea-Fernández et al., 2018; Hurtado et al., 2013; Rivera et al., 2016). As a result, breeders are now focusing more efforts into improving internal

content of their varieties in order to please consumer's preferences. However, improving fruit internal content is a tremendously difficult task because modern varieties have evolved into a diversity bottleneck that completely altered its metabolomics and, because of the polymorphic control of quality traits, significant advances in this area are slow and laborious (Casals et al., 2011; Egea-Fernández et al., 2018).

Since peppers are usually poor in sugars and organic acids, their taste is mostly dependent on their content in both capsaicinoids, responsible for the pungent taste, and volatiles, responsible for the characteristic flavour (Bosland and Votava, 2012; Moreno-Peris et al., 2012; Parisi et al., 2017; Rodríguez-Burruezo et al., 2010, 2006). Hence, those varieties that are consumed as a vegetable should be improved towards higher contents of these two compounds and for other health-promoting elements, such as antioxidants and vitamins, in order to satisfy consumer's demands. However, for those fruits which the main purpose is to be used a food colorant or spice, efforts should be directed towards improving the concentration of capsaicinoids and carotenoids, pigment responsible for fruit colour (Bosland and Votava, 2012; Moreno-Peris et al., 2012; Parisi et al., 2017; Rodríguez-Burruezo et al., 2010, 2006).

Within internal quality parameters, pungency has been the dominant subject in pepper breeding, as numerous reports can accredit for (Blum et al., 2013; Kim et al., 2014; Nimmakayala et al., 2016a; Rodríguez-Burruezo and Nuez, 2006; Zewdie and Bosland, 2000). In Spain, CITA has been the reference institution regarding capsaicinoids heritage and breeding (Fayos et al., 2017; Garcés-Claver et al., 2007; Olguín-Rojas et al., 2019; Rodríguez-Maza et al., 2012). Furthermore, pepper's use as food colorant and spice has been significantly studied and there are numerous varieties developed with this purpose, such as Spanish PDOs 'Pimentón de Murcia' and 'Pimentón de la Vera' (Nuez et al., 2003; Rodríguez-Burruezo et al., 2016). On that regard, pepper carotenoids are a family of red, orange and yellow pigments responsible for fruit colour and represent the main target in pepper breeding programs for higher and stable colorant power, and are widely used by food, cosmetics and pharmaceutical industries (Arimboor et al., 2015; Gómez-García and Ochoa-Alejo, 2013; Lerma et al., 2014; Rodríguez-Burruezo et al., 2009; Sánchez et al., 2015). In addition, these compounds have recognized beneficial properties to human health, as antioxidants and vitamin precursors, which make them interesting also in varieties consumed fresh (Fiedor and Burda, 2014). The Spanish institution IMIDA has been the main reference regarding pepper breeding for colorant power and has established, recently, a collaboration with COMAV, in order to improve these compound's stability against oxidation in the post-production phase (Fita et al., 2014; Sánchez et al., 2015).

Despite having a major role in pepper's flavour, volatile content has been given little attention in modern pepper breeding programs, and only in the last decade these compounds have been intensively studied (Parisi et al., 2017; Rodríguez-Burruezo and Nuez, 2006). For example, work developed by the *Capsicum* breeding group of the

COMAV has shed light into the volatile fraction of several *Capsicum* materials, into the inheritance of these compounds, and enabled the identification of more than 100 new compounds, paving the way for the introgression of said properties into other cultivars (Moreno-Peris et al., 2012; Rodríguez-Burruezo et al., 2010). Other works have also reported an incredibly variable number of volatiles in pepper, ranging from 63 up to 254 different compounds, depending on species, variety, ripening stage and cultural practices, suggesting the possibility of creation of new combinations of volatiles and new aromas (Eggink et al., 2012; Luning et al., 1994; Moreno-Peris et al., 2012; Parisi et al., 2017; Pino et al., 2006; Rodríguez-Burruezo et al., 2010).

Pepper's nutritional value is not limited to the compounds mentioned above, in fact, there are several other metabolites and elements relevant to human diet present in pepper pods. In addition, pepper pods are a great source of vitamins and polyphenols (Pellegrini et al., 2003; Rodríguez-Burruezo et al., 2013b, 2009; Sarpras et al., 2019). Regarding vitamins, *Capsicum* fruits provide essentially vitamins A, C and E, which have important roles in the human metabolism and help prevent several health conditions (Rodrigo et al., 2014; Sarpras et al., 2019; Smirnoff and Wheeler, 2000; Yahia et al., 2019). In fact, most of pepper's antioxidant activity is due to the vitamin C concentration, also known as L-ascorbic acid, which could represent up to a quarter of the total amount of antioxidant activity in pepper (Chu et al., 2002), and it is not unusually to find materials that are able to provide the daily recommended dose of vitamin C with less than 100 g portion, far more than other vegetable and fruits known for their vitamin C content (Morales-Soto et al., 2014; Pellegrini et al., 2003; Ribes-Moya et al., 2018; Rodríguez-Burruezo et al., 2013a, 2011). Furthermore, a considerable amount of variability has been described for this trait, indicating a strong genotype, environmental and ripening stage effects, although researchers seem to agree that mature fruits have higher vitamin C concentrations (Pérez-López et al., 2007a; Ribes-Moya et al., 2018; Rodríguez-Burruezo et al., 2013a, 2011). As a result, breeding for high content in vitamin C has been a secondary goal although it is still measured as a relevant indicator of nutritional quality (Rodríguez-Burruezo and Nuez, 2006).

Pepper pods are rich in polyphenols, which are secondary metabolites involved in several plant mechanisms, including defense and reproduction, and pepper encompasses a wide range of these compounds which are becoming increasingly desired due to their antioxidative activity (Anantharaju et al., 2016; Gould, 2004; Landi and Tattini, 2015; Pourcel et al., 2007; Yahia et al., 2019). Thus, peppers usually rank high in terms of phenolic content, comparing to other rich vegetables, especially pungent types, because capsaicinoids are phenolic compounds (Ribes-Moya et al., 2018; Rodríguez-Burruezo et al., 2009). Plus, previous reports have detected a wide variation for phenolic content among varieties and species, being those effects more important than cultural practices or stress on phenolic concentration (Ribes-Moya et al., 2018; Rodríguez-Burruezo et al., 2009; Russo, 2012). In pepper, the most frequent phenolic compounds found are

flavonoids and, within these family of compounds, flavanol and flavone are the most relevant (Chu et al., 2002; Rodríguez-Burruezo et al., 2009; Rodríguez-Burruezo and Nuez, 2006). In addition, another important flavonoid that starts to receive attention is anthocyanin which has an important part in the plant's response to biotic and abiotic stresses as well as a health-promoting benefits humans (Gould, 2004; Landi and Tattini, 2015).

Finally, pepper is also a good source of macro and microminerals (Pérez-López et al., 2007b; Rubio et al., 2002; Sarpras et al., 2019). However, contrarily to some of the health-promoting compounds mentioned above, pepper pods mineral content has only been studied for a reduced number of varieties and *C. annuum* concentrated most of those efforts (Pérez-López et al., 2007b; Rubio et al., 2002). We believe that the study of the mineral profile of a wider fraction of *Capsicum* germplasm would provide information to researchers of which materials may be fit to use in breeding programs for improved fruit internal content or to be introduced directly into the market as high quality varieties.

As mentioned above, important achievements have been made towards the characterization of peppers internal content and how it may affect human health (Yahia et al., 2019). However, after a long period of neglection, improvement of cultivars nutritional and organoleptic content is going to need a major effort from researchers and breeders (Casals et al., 2011; Egea-Fernández et al., 2018). In order to restore the mechanisms controlling accumulation of health-promoting compounds, the scientific community needs, first, to find sources of variation for those characters and, second, to characterize the metabolic routes controlling those traits (Parisi et al., 2017; Ribes-Moya et al., 2018; Rodríguez-Burruezo et al., 2009). On that matter, exploiting the natural variability within landraces, as well as, within related species from different germplasm pools may enlighten the way to discover new compounds, new combinations of compounds and, to increase their concentration in other cultivars (Parisi et al., 2017; Ribes-Moya et al., 2018; Rodríguez-Burruezo et al., 2009). On that regard, *C. baccatum* is known as a remarkable genetic pool for several traits of interest and is an asset of unexplored variability and richness in bioactive compounds (Rodríguez-Burruezo et al., 2009; Scaldaferro et al., 2018; Yoon et al., 2006; Zonneveld et al., 2015). The introduction of this species in breeding programs could be a huge step towards the improvement of pepper's internal quality. In addition, genotype × environment and genotype × maturity stage interactions studies are crucial in order to understand how a certain genotype behaves under specific conditions, and how its internal content varies with those effects. Thus, more studies regarding this interaction are needed so breeders can select the fitter individuals and with the best organoleptic quality (Figàs et al., 2018a; Ribes-Moya et al., 2018; Rodríguez-Burruezo et al., 2009).

Finally, improving the organoleptic and nutritional quality of a crop does not only provides benefits to human health, in addition to that, improved varieties would have a higher resilience to biotic and abiotic stresses, since several of these compounds have a protective effect (Gould, 2004; Landi and Tattini, 2015; Luna-Ruiz et al., 2018; Pourcel et al., 2007; Smirnoff and Wheeler, 2000) and, at the same time, we would be developing added-value varieties with a good acceptance by consumers (Casals et al., 2011; Egea-Fernández et al., 2018; Parisi et al., 2017; Rivera et al., 2016; Rodríguez-Burruezo et al., 2009; Rubio et al., 2002).

7| *Capsicum* phenomics and genomic tools

7.1 Phenomics: state of the art

A lot has changed since the XVIth century botanists' Era, in terms of germplasm characterization. On one hand, first botanists had limited access to specimens, since often they had access only to herbarium collections. And two, characterization relied on a reduced set of morphological traits (Andrews, 1995). Currently, this is no longer an issue, since *ex situ* collections can be easily accessed by researchers all around the world, throughout hundreds of genebanks (AVRDC, 2019; CGN, 2019; Díez et al., 2018; USDA et al., 2019) and, nowadays, almost every relevant crop in the world has a set of standardized descriptors that encompass almost every aspect regarding taxonomy to cultivation (Gotor et al., 2008). Hence, the development and implementation of descriptors was such a crucial mark for crop germplasm management that it is still used today (Bioversity International, 2019; UPOV, 2019). In fact, availability of internationally recognized and highly heritable descriptors throughout the scientific community is of paramount importance, since it enables the comparison of results and increases accuracy of drawn conclusions (Gotor et al., 2008). Therefore, crop breeding relies, in great measure, on the collected phenotypic data and on the accuracy of that data in order to describe variability regarding fruit (Parisi et al., 2017), leaves (Guijarro-Real et al., 2019), root (Bui et al., 2015), yield (Figàs et al., 2018a), plant physiology (Fullana-Pericàs et al., 2018), resistances (Chhapekar et al., 2018; Silvar and García-González, 2017), among other traits.

Despite the numerous advantages of standardised descriptors, morphological characterization is still a tedious task, for the most part performed manually, where descriptors often result ambiguous, difficult to evaluate or do not fully translate the character's nature or range (Brewer et al., 2006; Figàs et al., 2018b). In addition, conventional characterization is highly dependent on the collector's experience, which may lead to significant variation when different personal performs the characterization task. Furthermore, scientific knowledge and technological advances made possible to analyse increasing amounts of data, enabling an exhaustive characterization of even complex traits (Figàs et al., 2018a; Paez-Garcia et al., 2015; Tripodi and Greco, 2018). Hence, there is an urgent need for fast, objective and accurate methods in order to enable the collection of the maximum information possible per phenotyping exercise in a non-destructive manner (D'Agostino and Tripodi, 2017). In fact, phenotyping has become the bottleneck in crop breeding in the era of high-throughput genomics, thus the urge to dispose of fast and accurate information on phenotype in order to link those traits to genomic regions to fully exploit available resources (D'Agostino and Tripodi, 2017; Furbank, 2009).

During the last years, a plethora of tools has been developed, resulting in a wide range of applications that perform analysis from fruit to root, and from plant organs to cellular organelles (Lobet et al., 2013). Most of these tools rely on automated technologies and non-invasive methods to analyse plant traits by digital imaging. Hence, within those methods, we may find different systems and technologies depending on the target of characterization. For, example, infrared thermography has been applied for abiotic stress tolerance, such as salt and heat, near-infrared imaging has been used for leaf area calculation, far-infrared imaging to study shoot temperature and grain infestation, fluorescence imaging for assessing plant photosynthetic performance, X-ray to measure grain quality, among others (Furbank and Tester, 2011; Yang et al., 2013). However, almost all are only present in industrial crops (D'Agostino and Tripodi, 2017).

On that matter, one of the most successful phenomics tools is Tomato Analyzer, developed by Esther van der Knaap Laboratory of Ohio State University (Brewer et al., 2006). Tomato Analyzer software is a high-throughput phenomics software that provides fast, accurate and semi-automatic measurements of more than 40 fruit traits that are otherwise impossible to obtain manually (Brewer et al., 2007, 2006; Darrigues et al., 2008; Gonzalo et al., 2009; Gonzalo and van der Knaap, 2008; Rodríguez et al., 2010a, 2010b). Initially developed for tomato fruit shape characterization and QTL detection, this software was readily introduced in other crops and plant organs phenotyping, due to its objectivity and accuracy, being now widely used (Brewer et al., 2007; Darrigues et al., 2008; Figàs et al., 2014; Guijarro-Real et al., 2019; Hurtado et al., 2013; Plazas et al., 2014; Tripodi and Greco, 2018). In addition to the precise measurements of several parameters, Tomato Analyzer is freely available, and requires only a scanner connected to a computer with basic specs to be able to perform the characterization. Furthermore, the team responsible for its development keeps updating the programs abilities and performance (Ramos et al., 2018). Tomato Analyzer phenotyping utility has been widely demonstrated in the recent years, by detecting subtle differences among tomato and eggplant landraces (Figàs et al., 2014; Hurtado et al., 2013), by enabling the study of heterosis of agronomic traits of hybrids between eggplant and its wild relatives (Kaushik et al., 2016), study the morphologic diversity of *Capsicum* species (Tripodi and Greco, 2018), study heritability of fruit traits of pepper (Naegele et al., 2016), and detection of QTLs associated to fruit traits (Colonna et al., 2019).

Hence, the development of high-throughput phenotyping tools has a major impact on plant breeding (Yang et al., 2013). However, the high cost and the lack of automatization in the analysis of huge amounts of collected data are relevant issues that scientists still have to address in order to make it as normal as, for example, molecular methods in the majority of research institutions (White et al., 2012). Only then, phenomics will be able to provide all of its potential to breeders and be able to respond in an efficient manner to the necessities of crop breeding programs, especially regarding complex traits.

7.2 *Capsicum* genomics in the high-throughput era

Throughout the last decade we have been seeing unprecedented developments in genetic studies, and particularly within genomics, driven by the need for low cost data and faster analysis tools (Glaubitz et al., 2014; He et al., 2014). These changes were so significant that we went from using a couple dozen markers to using several thousands in a single experiment, in just a few years (Colonna et al., 2019; Naegele et al., 2016). Those advances are of paramount importance since plant breeding is highly dependent on the availability of informative genetic tools, such as molecular markers, in order to produce rapid and accurate results (Lee, 2019).

Regarding that, molecular and biochemical markers have been widely used in plant breeding since the 1980-90's, due to both their ability to in-depth characterize and to enable efficient selection of interesting materials through marked-assisted selection (MAS) (He et al., 2014; Moury et al., 2000). That is also true for *Capsicum* species, which had their first interspecific genetic linkage map published in 1998, using a battery of restriction fragment length polymorphisms (RFLPs), during the first generations of molecular markers (Tanksley et al., 1988). Since then, several works have published new interspecific maps using different varieties/species and several segregating populations (F_2, BC, RILs), in order to study heredity traits (Lee, 2019). Furthermore, introduction of new types of molecular markers and construction of several integrated genetic linkage maps, increased map saturation and enabled more accurate dissection of QTLs (Lefebvre et al., 2002, 1995; Paran et al., 2004). In fact, those maps enabled the first evaluation of synteny among different Solanaceae (Tanksley et al., 1988). As a result, researchers have now at their disposal a remarkable variety of well documented and defined tools from which to choose accordingly to their goals or limitations for a series of applications such as genetic diversity, population structure analysis and MAS (Lee, 2019).

Naturally, molecular techniques have evolved, since then, to cheaper, faster and more informative methodologies, and SNPs have become the preferred tool of researchers, due to their flexibility, reduced error rate, effectiveness, remarkable abundance, and wide distribution along the genome (Mammadov et al., 2012). Thus, the application of SNPs in *Capsicum* has been widely adopted as well as proven useful for genetic diversity, population structure studies (Lee et al., 2016) and mapping and QTL detection (Cheng et al., 2016).

In the recent years, technology has made possible for researchers to develop more complex projects at a fraction of the cost. As a result, the scientific community aimed efforts at developing high-quality genomes for every important organism in the world, in order to enhance knowledge about relevant evolutionary and biological processes (Genome 10K Community of Scientists, 2009; McCouch et al., 2013). For *Capsicum*,

that goal was achieved in 2014 when *C. annuum* cv. Serrano Criollo de Morelos 334 (CM334) and *C. chinense* PI159236 genomes were made public by an international research team (Kim et al., 2014). Impressively, within that same year, another international collaboration published two new pepper genomes, *C. annuum* cv. Zunla-1 and *annuum* wild relative, *C. annuum* var. *glabriusculum* (Qin et al., 2014). In addition, further developments regarding pepper genomic resources were seen just a few years later, when *C. baccatum* PBC81 and updated versions of CM334 and PI159236 genomes were reported by the same team that had sequenced and assembled the first two pepper genomes (Kim et al., 2017). Lastly, Hulse-Kemp and her team published the genome of accession UCD-10X-F1 (F_1 Hybrid), resulting from a cross between CM334 and non-pungent blocky breeding line (Hulse-Kemp et al., 2018).

Availability of high-quality reference genome is a powerful resource for plant breeding that enables the study of the genomic structure underlying all biological mechanisms regarding an entire taxa (Qin et al., 2014). In addition, it enables the study of evolutionary processes and the understanding of genomic elements function. Ultimately, it enables precise gene and gene function dissection to be then introgressed into other individuals and exploited by breeders. Likewise, the study of the first pepper genomes provided important insights into its evolution and structure at an unprecedent level of detail. As a result, researchers concluded that the unusually large genome size (~3.5Gb) and degree of repetitiveness (~80%), compared to other Solanaceae, was the result of several duplication phenomenon before speciation, within both euchromatin and heterochromatin regions, mainly due to long terminal repeat transposable elements of the *Gypsy* type (Kim et al., 2014; Qin et al., 2014). In addition, researchers were able to confirm the importance of tandem duplication events in the origin of new gene functions (Kim et al., 2017). In the case of pepper, several of these events shaped the acyltransferase gene family, with important role controlling the pungency trait (Kim et al., 2014; Qin et al., 2014). Whole genome resequencing has been successfully used in order to close knowledge gaps regarding genomic transformations due to domestication and breeding processes, to identify and fine mapping genomic regions linked to traits of interest (Gramazio et al., 2019; Tripodi et al., 2019), and even to reconstruct pepper landraces genomes (Barchi et al., 2017). Despite these developments, *Capsicum* molecular tools still lag behind other important Solanaceae, such as the ones of tomato or potato (Ashrafi et al., 2012; Hulse-Kemp et al., 2016).

At the same time that *Capsicum* genomic tools have been improving, high-throughput next generation sequencing tools (NGS) have also been immensely improved and are now accessible to the majority of research laboratories (Elshire et al., 2011; Poland and Rife, 2012). Several sequencing technologies have been developed and used with remarkable utility for crop breeding and genetic studies, providing fast genome-wide analysis and development of markers (D'Agostino and Tripodi, 2017; Lee, 2019). Regarding those, genotyping-by-sequencing (GBS) had an important impact on the scientific community, and is probably the most adopted genotyping strategy at the

moment, due to its versatile application (Elshire et al., 2011; Poland and Rife, 2012; Scheben et al., 2017; Sonah et al., 2013). Hence, GBS is a low-cost genome-wide genotyping method that enables SNP discovery, high-density genetic mapping, GWAS, and genomic selection independently of the target species, population, or the availability of previous genomic information (Elshire et al., 2011; Poland and Rife, 2012).

Regarding *Capsicum* genomic studies, the combination of reference genomes and GBS technology has successfully been implemented with impressive results (Lee, 2019). The combination of these tools enabled the rapid and accurate detection of thousands of highly informative genome-wide SNPs which are now available for researchers to use. In the last years, GBS-generated SNPs have been proven useful for detection of trait-associated QTLs (Ahn et al., 2018; Cheng et al., 2016; Nimmakayala et al., 2016a, 2016b), landrace evolution and differentiation process (Taitano et al., 2018), diversity and population structure analysis (Taitano et al., 2018), and even for the development of genotyping platforms (Hulse-Kemp et al., 2016).

We are advancing at a pace never seen before in plant breeding, thanks to the huge developments in genomic studies and technological advances. As we go further into the future, we are going to be able to understand more about the mechanisms underlying biological functions and, with that, being able to respond faster to adversities. Thus, future efforts should be focused on adaptation to low input conditions and resistance to biotic and abiotic stresses, in order to cope with climate change conditions. In addition, and since we are more capable now of studying complex traits, efforts should be made to increase both the nutritional content and taste of crops in order to satisfy consumer demand. The combination of genomic and phenomics is going to play an important part in order to achieve these goals.

Aims |

The main goal herein is to use both phenomics and genomic tools to characterize and improve *Capsicum* spp. germplasm, with particular emphasis on Spanish *C. annuum* landraces, ecotypes and heirlooms. Thus, we aspire to study their morphological variability, their phylogeny, their genetic structure, their relationship with other cultivars and specimens, their nutritional value, and their adaptation to abiotic stress conditions with the goal of exploiting the genotype per environment interaction as a mean to select elite materials for high productivity, nutritional value and adaptation to stress conditions.

Hence, in order to achieve the proposed goal, we planned the following five specific main objectives:

1. Morphological characterization and typification based on both conventional IPGRI descriptors for *Capsicum* and high-throughput phenotyping tool Tomato Analyzer, of highly representative and diverse *C. annuum* collection of traditional materials from the Spanish centre of diversity, in order to assess morphological diversity among and within varietal types, as well as, to identify highly discriminant traits and parameters among them towards varietal registration and conservation.

2. Assessment of morphological and genetic diversity within a collection of two families of *criollo* pepper landraces, from the Mexican centre of diversity, as well as, how does the open-pollination traditional breeding method affects both phenotypic uniformity and allele fixation in order to get insights into the development of materials adapted to climate change conditions encompassing a wider genetic diversity.

3. Molecular characterization and genomic fingerprint determination for ecotype differentiation, using genotyping-by-sequencing, in order to shed light into the origins of the Spanish materials, their phylogenetic relation with materials from other centres of diversity, and their genetic structure, as a first step towards the selection of relevant materials for the development of introgression lines and other experimental populations, such as MAGIC.

4. Characterization of a *Capsicum* spp. collection for its fruit internal content in health-promoting compounds, such as ascorbic acid and minerals, in order to select high quality materials, and adapted to the Mediterranean conditions, to be used as breeding lines for improving fruit quality of other varieties or to be used as a parental line in the construction of segregating populations.

5. Characterization of the main root adaptations to phosphorus low input conditions of a collection of *Capsicum* spp. materials in order to enhance our understanding of the mechanisms underlying the response as a first step towards the selection of elite materials with high resilience and high use efficiency to low input conditions as a climate change mitigation measure.

Results |

Chapter I: Morphological characterization of *Capsicum* spp.

Phenomics of elite heirlooms of pepper (*Capsicum* spp.) from the Spanish centre of diversity: conventional and high-throughput digital tools towards varietal typification

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Abstract

Spain is a relevant secondary centre of diversity for *C. annuum* peppers, especially for the bell types known as *Pimiento Morrón* or *Pimiento de Morro*. Thus, a myriad of highly regarded landraces adapted to a wide range of conditions can be found throughout the country, as a result of centuries of farmers breeding. Despite that, these materials lack of proper characterization and typification, of paramount importance for farmers, breeders and germplasm management. In this regard, in addition to internationally accepted conventional descriptors, new phenomics digital tools may provide a vital help towards exhaustive germplasm characterization. With this aim, 32 conventional descriptors and 35 digital traits were used herein to characterize a large collection of *C. annuum* accessions from all Spanish regions, including PDOs and PGIs, with emphasis in *Morrón* peppers, in order to assess the diversity within Spanish elite germplasm and to test the efficiency of those methods to differentiate varietal types and closely related materials. A considerable amount of variation was found using both conventional and digital descriptors, even within *Morrón* pepper groups, reflecting the diversity of Spanish peppers in terms of plant and fruit morphology, essential for future breeding programs. Both conventional descriptors and digital parameters were able to distinguish varietal groups. However, on the whole, digital phenotyping was able to discriminate in a more accurate way. Most digital parameters were able to discriminate varietal groups into higher numbers of categories (≥4) than conventional traits (usually 2-4). In addition, the number of significant pairwise differences among varietal groups was considerably higher for digital parameters than for conventional descriptors, enabling a powerful separation, particularly relevant for closely related groups such as *Morrón* peppers. Likewise, as revealed by PCA, digital phenotyping allowed a more powerful intra-varietal separation compared to conventional descriptors. Finally, a subset of 4 conventional descriptors and 13 Tomato Analyzer traits were identified as the most discriminant to distinguish among closely related *C. annuum* accessions, explaining 81.81% of total variance found by PCA. Fruit traits explained the highest percentage of variance for our collection.

Introduction

Native from America, peppers (*Capsicum* spp.) are one of the most popular vegetables, contributing with its flavour to a wide range of culinary specialities all around the world (Bosland and Votava, 2012). *Capsicum* is a small but genetically and morphologically diverse genus comprising five cultivated species and almost 40 wild species (Barboza et al., 2019; Carrizo García et al., 2016; Moscone et al., 2007). Among the cultivated species, *C. annuum* L. (var. *annuum*) is the most diverse and economically important species, and its cultivars are grown in almost all temperate and tropical regions of the world (Bosland and Votava, 2012; FAO, 2019).

Spain is a highly relevant secondary centre of diversity for *C. annuum*, since its introduction from America in the late XVth century (Andrews, 1995; González-Pérez et al., 2014; Nuez et al., 2003). Five centuries of cultivation and breeding have led to the bearing of a plethora of Spanish ecotypes adapted to a wide range of agro-climatic conditions (González-Pérez et al., 2014; Rivera et al., 2016; Rodríguez-Burruezo et al., 2016). As a consequence, many landraces, grown since immemorial times, can still be found nowadays in all Spanish regions, particularly those from varietal types known as *Pimiento Morrón*, *Morrón de Cascos* or *Pimiento de Morro* (*i.e.* resembling the nose of a cow, *Morro* in Spanish), encompassing sweet bell peppers (from blocky to rectangular shapes) with medium-large sized pods, as well as their round/heart-shaped relatives called *Morrón de Bola* or *Morrón de Conserva* (Rodríguez-Burruezo et al., 2016).

Furthermore, peppers hold the highest number of Protected Designations of Origin (PDOs) and Protected Geographical Indications (PGIs) among vegetables and food derivatives in Spain (MAPA, 2019). Ecotypes such as *Padrón-Herbón* (Galicia), *Bola* (Murcia; for *Pimentón de Murcia*), *Bierzo* and *Morrón de Fresno-Benavente* (Castilla y León), *Piquillo de Lodosa* (Navarra), *Jaranda* (Extremadura; for *Pimentón de la Vera*), *Riojano* (La Rioja), and *Guindilla de Ibarra* and *Gernika* (Basque Country), among others, are highly considered among consumers (Rivera et al., 2016; Rodríguez-Burruezo et al., 2016).

However, not all Spanish landraces benefit from being recognized with protected designations. Those that are not included in such groups are in high risk of genetic erosion due to its substitution by F_1 cultivars of California Wonder and Lamuyo types, highly productive and resistant to several pathogens, but encompassing a narrow genetic diversity (Lanteri et al., 2003; Rivera et al., 2016; Rodríguez-Burruezo et al., 2016). Consequently, the abandonment of ancient materials seriously threatens the agrodiversity (Brugarolas et al., 2009; Hammer, 2004; Hammer et al., 2003; Votava et al., 2005).

Fortunately, consumers are becoming increasingly interested in tastier foods produced in environmentally sustainable systems, and this situation offers a great opportunity for recovering the ancient cultivars while maintaining the farmer's source of income (Brugarolas et al., 2009; Casals et al., 2011; Hurtado et al., 2014; Parisi et al., 2017; Pérez-López et al., 2007; Rivera et al., 2016; Zonneveld et al., 2015). In fact, the demand for traditional varieties is increasing gradually and they even reach higher prices than those from modern varieties (Brugarolas et al., 2009; Casals et al., 2011). In this frame, it is essential to increase the added-value of landraces among consumers and to make efforts to characterize and to preserve such valuable resources *in situ*. Thus, an exhaustive characterization of cultivars is of paramount importance, especially for those which still lack typification (Lanteri et al., 2003; Parisi et al., 2017; Spataro and Negri, 2013). For instance, the popular term *Pimiento Valenciano* encompasses a wide range of relevant *Morrón* peppers from the Region of Valencia, but this denomination still lacks varietal typification (Rodríguez-Burruezo et al., 2016).

With this aim, morphological characterization based on standardized descriptors is an important practice for germplasm identification. The availability of an internationally recognized set of highly heritable descriptors throughout the scientific community enables the comparison of results as well as the characterization of cultivars (Bioversity International, 2017; Gotor et al., 2008; UPOV, 2019). However, these descriptors are sometimes tedious and often difficult to evaluate, particularly when differences between accessions are very subtle (Brewer et al., 2006; Costa et al., 2011; Figàs et al., 2018).

To this regard, Tomato Analyzer, a high-throughput phenomics software tool, provides fast, accurate and semi-automatic measurements of a large set of fruit traits that are otherwise impossible to obtain manually (Brewer et al., 2007, 2006; Darrigues et al., 2008; Gonzalo et al., 2009; Gonzalo and van der Knaap, 2008; Rodríguez et al., 2010a, 2010b). Despite being initially developed for tomato fruits characterization, it has been successfully used to characterize other crops (Darrigues et al., 2008; Hurtado et al., 2013; Naegele et al., 2016; Plazas et al., 2014).

On this matter, pepper germplasm still lacks large-scale phenomics characterization that could be used in parallel with the exponentially increasing available genomic information in order to fully exploit all the resources and unveil new favourable allelic combinations (Ashrafi et al., 2012; Hulse-Kemp et al., 2018, 2016; Kim et al., 2017, 2014; Park et al., 2012; Qin et al., 2014; Zonneveld et al., 2015). The use of both conventional and Tomato Analyzer descriptors might lead to a more detailed and powerful morphological characterization of pepper varieties resulting in a better separation of closely related materials.

Here we present the morphological characterization, using a set of conventional descriptors and Tomato Analyzer parameters, of a large collection of pepper accessions that includes a comprehensive representation of heirlooms and landraces from all the

Spanish regions. To our knowledge, this is the first work to use conventional and phenomics tools to characterize such a large collection of peppers from the relevant Spanish centre of diversity. Our goals were: i) to assess the morphological diversity of the Spanish pepper landraces in order to contribute to varietal typification, promotion and preservation, and ii) to estimate the discrimination power of both conventional descriptors and phenomics software, separately and combined, particularly for highly close materials.

Material and methods

Plant material and growing conditions

A collection of 109 pepper accessions, encompassing 106 *C. annuum* accessions and other species from the *annuum* complex, i.e. *C. chinense* (2) and *C. frutescens* (1), was characterized (Table 1). This collection is representative of the most relevant heirlooms and landraces from the Spanish centre of diversity, with special emphasis on the highly appreciated *Morrón* peppers, as well as other foreign materials as controls. All the regions of Spain and 14 different countries were represented, as well as three varietal status (traditional, commercial and experimental lines) and nine main groups based on varietal assignment (Figure 1). These materials are maintained at the COMAV Germplasm Bank (Universitat Politècnica de València) and at the COMAV *Capsicum* breeding group and are the result of several collection expeditions over the past four decades (Table 1).

Five plants per accession were grown under mesh greenhouse and natural soil conditions, during the spring-summer of 2015, at the COMAV experimental fields (UPV Vera Campus, GPS coordinates: 39°28'56.33"N; 0°20'10.88"W). Transplanting was done in May at the five leaves stage, and fruit harvest was carried-out from July to October. Plants were spaced 1 m between rows and 0.50 m within the row, following a completely randomized design. Individual plants were trained with vertical strings, drip irrigated and pruned accordingly to the standard local practices for this crop. Phytosanitary treatments against whiteflies, spider mites, aphids and caterpillars were applied accordingly to their population levels.

Table 1 - List of accessions and corresponding abbreviation, name, origin, and varietal status.

Abbreviation	Accession: Local name (bank code)	Origin	Varietal type
Group I: *Morrón de cascos* (MC) Pochard´s groups A and B – Blocky and rectangular shape, medium to large size			
MC-1	Pimiento morro de vaca (BGV-57)	Huesca, Spain	Traditional
MC-2	Pimiento cuatro cascos (BGV-637)	Granada, Spain	Traditional
MC-3	Pimiento morrón (BGV-1319)	Asturias, Spain	Traditional
MC-4	Pimiento morrón (BGV-1814)	Tarragona, Spain	Traditional
MC-5	Cuatro morros (BGV-1834)	Barcelona, Spain	Traditional
MC-6	Morro de Vedella (BGV-1844)	Cataluña, Spain	Traditional
MC-7	Largo de Reus (BGV-1862)	Barcelona, Spain	Traditional
MC-8	Pimiento gordo de asar (BGV-4036)	Cáceres, Spain	Traditional
MC-9	Pimiento grueso de Murcia (BGV-4322)	Murcia, Spain	Traditional
MC-10	Morro de vaca (BGV-4329)	Murcia, Spain	Traditional
MC-11	Pimiento trompa de vaca (BGV-4348)	Murcia, Spain	Traditional
MC-12	Pimiento morro de vaca (BGV-4349)	Murcia, Spain	Traditional
MC-13	Pimiento cuatro cantos (BGV-5035)	Valencia, Spain	Traditional
MC-14	Pimiento cuatro cantos (BGV-5057)	Castellón, Spain	Traditional
MC-15	Pimiento gordo (BGV-5083)	Castellón, Spain	Traditional
MC-16	Trompa de vaca (BGV-5109)	Alicante, Spain	Traditional
MC-17	Morrón cuatro cantos (BGV-5113-1)	Alicante, Spain	Traditional
MC-18	Pimiento de Infantes (BGV-10368)	Ciudad Real, Spain	Traditional
MC-19	Pimiento de casco (BGV-10540)	Albacete, Spain	Traditional
MC-20	Pimiento cuatro morros (BGV-10599)	León, Spain	Traditional
MC-21	Largo de Reus (BGV-10600)	Tarragona, Spain	Traditional
MC-22	Morrón de cuatro Picos (BGV-10946)	Asturias, Spain	Traditional
MC-23	Pimiento morro de vaca (BGV-11038)	Albacete, Spain	Traditional
MC-24	Pimiento de cuatro morros (BGV-11213)	Cantábria, Spain	Traditional
MC-25	Pimiento morrón largo (BGV-11267)	León, Spain	Traditional
MC-26	Morrón de Loyola cuatro cantos (BGV-11528)	Guipúzcoa, Spain	Traditional
MC-27	Pimiento gordo de ensalada (BGV-11558)	Cáceres, Spain	Traditional
MC-28	Pimiento morrón gordo (BGV-11630)	Vizcaya, Spain	Traditional
MC-29	Pimiento gordo morro de vaca (BGV-11751)	Huesca, Spain	Traditional
MC-30	Pimiento gordo (BGV-13636)	Salamanca, Spain	Traditional
MC-31	Pimiento gordo (BGV-13638)	Zamora, Spain	Traditional
MC-32	California Wonder (red)	COMAV, Valencia, Spain	Experim. line
MC-33	California Wonder (yellow)	COMAV, Valencia, Spain	Experim. line
MC-34	De Infantes	Ciudad Real, Spain	Traditional
MC-35	De Infantes	Mascarell Seeds, Spain	Com. heirloom
MC-36	Largo de Reus	Barcelona, Spain	Traditional
MC-37	Largo de Reus	Mascarell Seeds, Spain	Com. heirloom
MC-38	Morrón de Fresno de la Vega y Benavente P.G.I.	León, Spain	Traditional
MC-39	Pimento de assar	Aveiro, Portugal	Traditional
MC-40	Tendre de Châteaurenard	F. Jourdan, INRA-GEVES, France	Traditional
MC-41	Carmagnola giallo	Carmagnola, Pierdmont, Italy	Traditional
MC-42	Carmagnola rosso	Carmagnola, Pierdmont, Italy	Traditional
MC-43	Cuneo Giallo	Franchi Sementi, Italy	Com. heirloom
MC-44	Giallo D'Asti	Franchi Sementi, Italy	Com. heirloom
MC-45	Peperone Cuneo giallo	Cuneo, Italy	Traditional
MC-46	Atina	Serbia	Com. heirloom
Group II: Valenciano (MV) Pochard´s groups B1 and B2 – Rectangular shape, large size			
MV-1	Valenciano (BGV-4331)	Murcia, Spain	Traditional
MV-2	Valenciano (BGV-5030)	Valencia, Spain	Traditional
MV-3	Valenciano (BGV-5103)	Valencia, Spain	Traditional
MV-4	Valenciano (BGV-5113 (2))	Alicante, Spain	Traditional
MV-5	Valenciano (BGV-5121)	Alicante, Spain	Traditional
MV-6	Valenciano (BGV-5126)	Alicante, Spain	Traditional
MV-7	Pimiento valenciano (BGV-10582)	Valencia, Spain	Traditional
MV-8	Valenciano	Valencia, Spain	Traditional

Table 1 (continuation) - List of accessions and corresponding abbreviation, name, origin, and varietal status.

Abbreviation	Accession: Local name (bank code)	Origin	Varietal type
MB-1	Pimiento morrón de bola (BGV-60)	Zaragoza, Spain	Traditional
MB-2	Pimiento morrón de conserva (BGV-614)	Jaén, Spain	Traditional
MB-3	Morrón de conserva (BGV-4335)	Murcia, Spain	Traditional
MB-4	Morrón de conserva (BGV-5041)	Valencia, Spain	Traditional
MB-5	Morrón de conserva (BGV-5114 (1))	Alicante, Spain	Traditional
MB-6	Morrón de conserva (BGV-5114 (2))	Alicante, Spain	Traditional
MB-7	Pimiento del País (BGV-10447)	Baleares, Spain	Traditional
MB-8	Lora (BGV-11500)	León, Spain	Traditional
MB-9	Pimiento morrón de conserva (BGV-11881)	Guadalajara, Spain	Traditional
MB-10	Calahorra	La Rioja, Spain	Traditional
MB-11	Bulgarski Ratund	Maritsa VCRI, Bulgaria	Traditional
MB-12	Topepo rosso	Italy	Traditional
MB-13	Topepo rosso	Franchi Sementi, Italy	Com. heirloom
Group IV: other thick flesh peppers Pochard´s groups C2 and C3 – Triangular shape, medium to large size			
IV-1	Pimiento grueso del país (BGV-4507)	Cantabria, Spain	Traditional
IV-2	Pimiento Najerano gordo (BGV-10451)	La Rioja, Spain	Traditional
IV-3	Pimiento gordo najerano (BGV-11092)	La Rioja, Spain	Traditional
IV-4	Pimiento de asar gordo najerano (BGV-13004)	Vizcaya, Spain	Traditional
IV-5	Pimiento de asar najerano (BGV-13009)	Vizcaya, Spain	Traditional
IV-6	Bierzo, Cons. Reg. P.G.I. Pimiento Asado Bierzo	León, Spain	Traditional
IV-7	Najerano	Ramiro Arnedo Seeds, Spain	Com. heirloom
Group V: Ancho/Piquillo peppers Pochard´s group C4 – Triangular shape, small size			
V-1	Pimiento de Pico (BGV-10183)	Navarra, Spain	Traditional
V-2	Pimiento del Piquillo (BGV-10186)	Navarra, Spain	Traditional
V-3	Pimiento del Piquillo, Cons. Reg. P.D.O.	Navarra, Spain	Traditional
V-4	Ancho 101	Reimer Seeds, Mexico/USA	Com. heirloom
V-5	Ancho mulato	Mexico	Traditional
Group VI: Cayenne/Guindilla			
VI-1	Guindilla (BGV-11531)	Guipúzcoa, Spain	Traditional
VI-2	Guindilla de Ibarra	S. Larregla, NEIKER, Spain	Traditional
VI-3	Torpedo de Bangalore	Bangalore, India	Traditional
VI-4	Chile de árbol	Mexico	Traditional
VI-5	Pasilla bajío	Mexico	Traditional
VI-6	Ka 2	Sri Lanka	Com. heirloom
VI-7	Rashi Bonnet	Sri Lanka	Traditional
VI-8	Ací Sivri	Turkey	Traditional
Group VII: Numex and Padrón Pochard´s groups B2, B4 and C2 – Elongated (medium to large size) and rectangular (small size)			
VII-1	Pimiento de Padrón (BGV-10185)	La Coruña, Spain	Traditional
VII-2	Pimiento de Padrón (BGV-11205)	Navarra, Spain	Traditional
VII-3	Pimiento de Herbón-Padrón P.D.O.	Galicia, Spain	Traditional
VII-4	Arnoia, P.G.I. Pemento da Arnoia	Galicia, Spain	Traditional
VII-5	Gernika cv. Derio, P.G.I. Gernikako Piperra	S. Larregla, NEIKER, Spain	Traditional
VII-6	Kapiya UV	Maritsa VCRI, Bulgaria	Traditional
VII-7	Sivriya 600	Maritsa VCRI, Bulgaria	Traditional
VII-8	Espelette P.G.I.	F. Jourdan, INRA-GEVES, France	Traditional
VII-9	Pettit Marsellais	F. Jourdan, INRA-GEVES, France	Traditional
VII-10	Poivre rouge de Bresse	F. Jourdan, INRA-GEVES, France	Traditional
VII-11	Peperone di Senise P.G.I.	Potenza, Senise, Italy	Traditional
VII-12	Numex Conquistador	New Mexico, USA	Traditional
VII-13	Chimayó	P.W. Bosland, New Mexico, USA	Traditional
VII-14	Numex Big Jim	P.W. Bosland, New Mexico, USA	Traditional
Group VIII: Jalapeno			
VIII-1	Chile Serrano	Mexico	Traditional
VIII-2	Jalapeno Candelaria	P.W. Bosland, New Mexico, USA	Traditional
VIII-3	Jalapeno Espinalteco	P.W. Bosland, New Mexico, USA	Traditional
VIII-4	Jalapeno M	Reimer Seeds, Mexico/USA	Com. heirloom
Group IX: Control/other Capsicums			
CON-1	Ají chirere (*C. frutescens*)	Venezuela	Traditional
CON-2	ECU-994 (*C. chinense*)	Equador	Traditional
CON-3	Habanero (*C. chinense*)	State College, Pennsylvania, USA	Traditional
CON-4	Pimiento de Bola, Cons. Reg. P.D.O. Pimentón Murcia	Murcia, Spain	Traditional

Figure 1 – Illustration of fruit diversity in the collection. Evaluated fruits and corresponding longitudinal cut indicate the common fruit morphology in each group.

Group I (A – California Wonder (MC-33) and B – Morrón de cuatro cascos (MC-2)), Group II (C – Valenciano (MV-6)), Group III (D – Morrón de Conserva (MB-4)), Group IV (E – Najerano (IV-7) and F – Pimiento de asar mucha carne (IV-5)), Group V (G– Piquillo de Lodosa (V-3)), Group VI (H – Guindilla de Ibarra (VI-2), I – Chile de árbol (VI-4) and J – Pasilla bajío (VI-5)), Group VII (K - Pimiento de Padrón (VII-3), L – Gernika (VII-5) and M – Kapiya UV (VII-6)), Group VIII (N – Jalapeno M (VIII-4) and O – Chile Serrano (VIII-1)), and Group IX (P - Habanero (CON-3).

Conventional characterization

Five individual plants per genotype were characterized using 32 conventional morphological descriptors for *Capsicum* related to plant (9), inflorescence/flower (7), fruit (15) and agronomic (1) traits (Table 2). Most descriptors corresponded to *Capsicum* descriptors from Bioversity International (IPGRI, 1995). In addition, other commonly used traits for germplasm characterization such as plant height, fruit weight, fruit colour, fruit cross-sectional shape, and yield were also considered. To avoid tedious reading of the manuscript as well as to make it more visual, all descriptors and traits were presented with abbreviations (Table 2).

Fruit colour was measured using a Minolta CR-300 colorimeter (Minolta Corporation, Osaka, Japan) and expressed accordingly to CIE L*a*b* 1976 colour space at the two commercial ripening stages of pepper, i.e. unripe and fully ripe. Two measures per fruit were taken at opposite sides of the equatorial region of the fruit. Colour parameters Chroma and HUE angle were then calculated as reported by Rodríguez-Burruezo et al. (2005) (Rodríguez-Burruezo et al., 2005). For descriptors involving measurements, four representative leaves (for mature leaf length and mature leaf width descriptors) and four representative fruits (for L*a*b* colour space coordinates) per plant were measured and values obtained for individual fruits were used to calculate the average value for each plant. Finally, yield per plant and average fruit weight were estimated at the end of the experiment considering all the commercial fruits per plant (Table 2).

Table 2 – List of conventional descriptors measured, corresponding abbreviation, IPGRI descriptor number, and units/scale.

Descriptor	Abbreviation	IPGRI Number	Units/scale
Plant descriptors			
Plant height	**PH**	-	cm
Growth habit	**GH**	7.1.2.7	3=Prostrate, 5=Intermediate (compact), 7=Erect
Nodal anthocyanin	**NA**	7.1.2.3	1=Green, 3=Light purple, 5=Purple, 7=Dark purple
Stem length	**SL**	7.1.2.9	cm
Branching habit	**BH**	7.1.2.11	3=Sparse, 5=Intermediate, 7=Dense
Leaf density	**LD**	7.1.2.13	3=Sparse, 5=Intermediate, 7=Dense
Leaf shape	**LS**	7.1.2.15	1=Deltoid, 2=Ovate, 3=Lanceolate
Mature leaf length	**MLL**	7.1.2.18	cm
Mature leaf width	**MLW**	7.1.2.19	cm
Inflorescence/Flower descriptors			
Number of flowers per axil	**FA**	7.2.1.2	1=One, 2=Two, 3=Three or more, 4=Many in bunches
Corolla colour	**CC**	7.2.1.4	1=White, 2=Light yellow, 3=Yellow, 4=yellow-green
Corolla spot colour	**CSC**	7.2.1.5	0=Absent, 1=White, 2=Yellow, 3=Green yellow, 4=Green, 5=purple
Anther colour	**AC**	7.2.1.8	1=White, 2=Yellow, 3=Light blue, 4=Blue, 5=Purple, 6=Dark purple
Flower position	**FP**	7.2.1.3	3=Pendant, 5=Intermediate, 7=Erect
Calyx margin	**CM**	7.2.1.15	1=Entire, 2=Intermediate, 3=Dentate
Calyx annular constriction	**CAC**	7.2.1.16	0=Absent, 1=Present
Fruit descriptors			
Fruit set	**FS**	7.2.2.4	3=Low, 5=Intermediate, 7=High
Fruit weight	**FW**	-	g
Fruit shape	**FSH**	7.2.2.7	1=Elongate, 2=Almost round, 3=Triangular, 4=Campanulate, 5=Blocky
Fruit surface	**FSUR**	7.2.2.19	1=Smooth, 2=semi wrinkled, 3=Wrinkled
Fruit cross-sectional shape	**FCSC**	-	1=Elliptic, 2=Rounded, 3=Quadrangular, 4=Triangular, 5=Irregular
Ripe fruit pungency (tasting)	**CAPS**	-	0=Absent, 1=Present
Anthocyanin spots or stripes	**AS**	7.2.2.2	0=Absent, 1=Present
Fruit shape at pedicel attachment	**FSPA**	7.2.2.13	1=Acute, 2=Obtuse, 3=Truncate, 4=Cordate, 5=Lobate
Fruit shape at blossom end	**FSBE**	7.2.2.15	1=Pointed, 2=Blunt, 3=Sunken, 4=Sunken and pointed
Exterior fruit colour lightness (unripe)	**Lu**	-	0=Black to 100=White
Exterior fruit colour Chroma (unripe)	**CHRu**	-	0=completely unsaturated to 100=fully saturated
Exterior fruit colour HUE (unripe)	**HUEu**	-	0°=red, 90°=yellow, 180°=green, 270°=blue
Exterior fruit colour lightness (ripe)	**Lr**	-	0=Black to 100=White
Exterior fruit colour Chroma (ripe)	**CHRr**	-	0=completely unsaturated to 100=fully saturated
Exterior fruit colour HUE (ripe)	**HUEr**	-	0°=red, 90°=yellow, 180°=green, 270°=blue
Agronomic descriptors			
Yield per plant	**Y**	-	g

Digital characterization

Four representative and commercially viable fruits per plant were longitudinally cut and scanned at a resolution of 300 dpi (dots per inch) with an Epson Expression 1640XL G650C scanner (Seiko Epson Corp., Japan). Stored images (TIF format) were then analysed using the Tomato Analyzer software (version 3.0) for 35 quantitative traits (Brewer et al., 2006; Darrigues et al., 2008; Rodríguez et al., 2010a). The descriptors measured included basic (7), fruit shape (3), blockiness (3), homogeneity (3), proximal fruit end shape (4), distal fruit end shape (4), asymmetry (6) and internal eccentricity (5) traits (Table 3). For blockiness, proximal fruit end shape, and distal fruit end shape descriptors default settings were used (Rodríguez et al., 2010a). Individual measures of each fruit were then used to obtain an average value for the corresponding plant (Table 3). As for the conventional descriptors and traits, all digital parameters were also given abbreviations.

Table 3 – List of digital phenotyping traits and parameters considered, and corresponding abbreviation, units, and brief description.

Trait	Abbreviation	Units	Description*
Basic			
Perimeter	**P**	cm	Fruit perimeter length.
Area	**AR**	cm^2	Fruit area.
Width Mid-height	**WMH**	cm	Measured at 1/2 of the fruit's height.
Maximum Width	**MW**	cm	Maximum horizontal distance of the fruit.
Height Mid-Width	**HMW**	cm	Measured at 1/2 of the fruit's width.
Maximum Height	**MH**	cm	Maximum vertical distance of the fruit.
Curved Height	**CH**	cm	Measured along a curved line through the fruit.
Fruit shape index			
Fruit Shape Index External I	**FSIE.I**	-	The ratio of maximum height to maximum width.
Fruit Shape Index External II	**FSIE.II**	-	The ratio of height mid-width to width mid-height.
Curved Fruit Shape Index	**CFSI**	-	The ratio of curved height to the width of the fruit at mid-curved-height.
Blockiness			
Proximal Fruit Blockiness	**PFB**	-	The ratio of the width at the upper blockiness position to width mid-height.
Distal Fruit Blockiness	**DFB**	-	The ratio of the width at the lower blockiness position to width mid-height.
Fruit Shape Triangle	**FST**	-	The ratio of the width at the upper blockiness position to the width at the lower blockiness position.
Homogeneity			
Ellipsoid	**ELL**	-	The ratio of the error resulting from a best-fit ellipse to the area of the fruit. Smaller values indicate more ellipsoid.
Circular	**CIR**	-	The ratio of the error resulting from a best-fit circle to the area of the fruit. Smaller values indicate more circular.
Rectangular	**RECT**	-	The ratio of the area of the rectangle bounding the fruit to the area of the rectangle bounded by the fruit.
Proximal fruit end shape			
Shoulder Height	**SH**	-	The ratio of the average height of the shoulder points above the proximal end point to maximum height.
Proximal Angle Micro	**PAMi**	degrees	The angle between best-fit lines drawn through the fruit perimeter on either side of the proximal end point.
Proximal Angle Macro	**PAMa**	degrees	The angle between best-fit lines drawn through the fruit perimeter on either side of the proximal end point.
Proximal Indentation Area	**PIA**	-	The ratio of the area of the proximal indentation to the total area of the fruit multiplied by 10.
Distal fruit end shape			
Distal Angle Micro	**DAMi**	degrees	The angle between best-fit lines drawn through the fruit perimeter on either side of the proximal end point.
Distal Angle Macro	**DAMa**	degrees	The angle between best-fit lines drawn through the fruit perimeter on either side of the distal end point.
Distal Indentation Area	**DIA**	-	The ratio of the area of the distal indentation to the total area of the fruit multiplied by 10.
Distal End Protrusion	**DEP**	-	The ratio of the area of the distal protrusion to the total area of the fruit multiplied by 10.

* For more detailed information about the descriptors check Rodríguez et al. (2010).

Table 3 (continuation) – List of digital phenotyping traits and parameters considered, and corresponding abbreviation, units, and brief description.

Trait	Abbreviation	Units	Description*
Obovoid	**OB**	-	Calculated as described by Rodríguez et al. 2010.
Ovoid	**OV**	-	Calculated as described by Rodríguez et al. 2010.
V. Asymmetry	**VA**	-	Average distance between a vertical line through the fruit at mid-width and the midpoint of the fruit's width at each height.
H. Asymmetry.ob	**HAOb**	-	Average distance between a horizontal line at mid-height and the midpoint of the fruit's height at each width.
H. Asymmetry.ov	**HAOv**	-	Average distance between a horizontal line at mid-height and the midpoint of the fruit's height at each width.
Width Widest Position	**WWP**	-	The ratio of the height at which the maximum width occurs to the maximum height.
Internal eccentricity			
Eccentricity	**ECC**	-	The ratio of the height of the internal ellipse to the maximum height.
Proximal Eccentricity	**PE**	-	The ratio of the height of the internal ellipse to the distance between the bottom of the ellipse and the top of the fruit.
Distal Eccentricity	**DE**	-	The ratio of the height of the internal ellipse to the distance between the top of the ellipse and the bottom of the fruit.
Fruit Shape Index Internal	**FSII**	-	The ratio of the internal ellipse's height to its width.
Eccentricity Area Index	**EAI**	-	The ratio of the area of the fruit outside the ellipse to the total area of the fruit.

* For more detailed information about the descriptors check Rodríguez et al. (2010).

Data analyses

Analysis of variance (ANOVA) was performed using individual plant values to identify significant differences among accessions as well as among varietal groups for both conventional and digital traits and parameters. To avoid scaling effects the ANOVA was performed using log transformed data (Figàs et al., 2014; Hills and Jackson, 1978). Student-Newman-Keuls post-hoc multiple range test was used to assess significant differences among cultivar groups. In addition, Principal Component Analysis (PCA) was carried out using Euclidean pairwise distances among accessions, first considering separately both conventional and digital characterizations and finally considering both sets of traits together. Statistics were carried out using STATGRAPHICS software (Statgraphics Centurion XVI, StatPoint Technologies, Warrenton, VA, USA) and plotted using the R package ggplot2 v2.2.1 (R Development Core Team, 2009; Wickham, 2016).

Results and discussion

Study of variation for the whole collection - *Conventional descriptors*

Highly significant differences ($P<0.001$ and $P<0.01$) were found for all conventional descriptors, with the only exception of CSC which was monomorphic for all accessions (Table 4). This was due to the fact that CSC is mainly used for species identification since it is present in *C. baccatum*, and other wild relatives, and consequently not useful for differentiation within *C. annuum* or related species, i.e. *C. chinense* and *C. frutescens* (DeWitt and Bosland, 1996; Moscone et al., 2007).

Most conventional descriptors showed considerable variation as can be observed in PH (24-207 cm), SL (5-60.20 cm), MLL (7.10-25 cm), MLW (2.30-14.60 cm), FW (0.54-353.67 g), Lu (33.00-74.89), CHRu (9.85-36.74), HUEu (99.76-148.75º), Lr (12.49-68.76), CHRr (11.60-45.36), HUEr (7.89-108.06º) or Y (127-3872 g) (Table 4). By contrast, other traits (mainly qualitative) showed a limited variation despite being polymorphic. This was particularly obvious for those traits with mean values close to one of the extremes of the range of variation, indicating that most accessions fit one of the categories of the descriptor (Table 4). This was the case of FA and CC (range of 1-4, but average close to 1), and CAPS and AS (usually absent). Such results were mainly due to the fact that most accessions belong to *C. annuum* and bell pepper (*Morrón*) type, and they usually present a single flower per axil, white corolla, and sweet fruits. Moreover, AS is an unwanted trait in ripe fruits, particularly for bell peppers, and farmers and breeders have usually performed selection against it. The low frequency of

anthocyanin spots in the fruits was also observed in another report regarding pepper landraces from Northern Spain (Rivera et al., 2016).

In terms of variation, our findings are in agreement with the high diversity reported for *C. annuum* (Bosland and Votava, 2012). In addition to the huge range of fruit shapes, we also found a remarkable variation for several traits that could have an important impact in future pepper breeding programs (Table 4). For example, the accumulation of carotenoids in the fruits is one of the most important traits of pepper, since they are an important component in the human diet (Fiedor and Burda, 2014; Rodríguez-Burruezo et al., 2009), and we found considerable variation for this trait among our accessions in terms of fruit colour parameters (Table 4). Finally, we also observed high diversity for both fruit weight and yield, which have paramount importance for the acceptance of the variety among farmers, retailers and consumers (Costa et al., 2011).

Study of variation for the whole collection - *Digital phenotyping*

Regarding the characterization based on phenomics tool, 33 traits showed highly significant differences ($P<0.001$) and two showed significant differences ($P<0.05$) for the whole collection. A wide range of variance was detected for most traits, with the most variable traits being P (8.53-53.63 cm), AR (3.19-129.51 cm^2), PAMi (0.40-358.19º), PAMa (1.09-352.04º), DAMi (0.55-358.33º) and DAMa (0.28-330.55º) (Table 4). Furthermore, mean values in most descriptors were close to the mid-value of the corresponding range, suggesting a wide diversity among the accessions evaluated (Table 4). Only a few traits such as fruit shape indexes (FSIE.I, FSIE.II and CFSI), distal fruit end shape descriptors (DEP), some asymmetry descriptors (VA, HAOb and HAOv) and one Internal eccentricity descriptor (FSII) showed mean values close to the lowest value of the corresponding range, indicating that a high number of accessions fit those values and therefore a low diversity among them. As found for conventional traits these findings were mainly due to limited variation among *Morrón de Cascos* and closely related types such as *Valenciano* and *Morrón de Bola* (Table 4).

These findings revealed an interesting level of diversity in our collection for both fruit size and shape and suggest a range of different shapes within *C. annuum* and even within the *Morrón* types which could have a direct application in future breeding programs in order to enhance the uses of these varieties. Size and shape of the fruits have a huge impact in several aspects, from farmers to consumer's preferences, in fact, they are the main attributes determining the packaging method and the acceptance of the variety (Costa et al., 2011).

Table 4 – Global means, ranges, and statistical significances of the conventional and digital traits and parameters considering the whole collection.

Conventional	Global mean/range [a]	Digital	Global mean/range [a]
Plant		***Basic***	
		P	33.20/8.53 - 53.63***
PH	103.84/24.00 - 207***	**AR**	52.06/3.19 - 129.51***
GH	5.09/3.00 - 7.00***	**WMH**	6.25/0.84 - 14.62***
NA	4.98/1.00 - 7.00***	**MW**	7.10/1.26 - 14.85***
SL	20.81/5.00 - 60.20***	**HMW**	8.16/1.25 - 17.13***
BH	4.61/3.00 - 7.00***	**MH**	10.17/2.93 - 18.70***
LD	4.83/3.00 - 7.00***	**CH**	10.91/3.05 - 20.47***
LS	1.63/1.00 - 3.00***	***Fruit shape index***	
MLL	14.90/7.10 - 25.00***	**FSIE.I**	1.76/0.45 - 9.95***
MLW	7.88/2.30 - 14.60***	**FSIE.II**	1.93/0.28 - 14.17***
		CFSI	2.56/0.51 - 16.88***
Flower		***Blockiness***	
		PFB	1.01/0.22 - 1.80***
FA	1.06/1.00 - 3.00***	**DFB**	0.61/0.25 - 1.09***
CC	1.08/1.00 - 4.00***	**FST**	1.79/0.30 - 4.19***
CSC	0.00/0.00 - 0.00 ns	***Homogeneity***	
AC	4.99/1.00 - 6.00***	**ELL**	0.11/0.05 - 0.23***
FP	6.27/3.00 - 7.00***	**CIR**	0.22/0.08 - 0.48***
CM	2.80/2.00 - 3.00***	**RECT**	0.49/0.09 - 0.68***
CAC	0.72/0.00 - 1.00***	***Proximal fruit end shape***	
		SH	0.08/0.00 - 0.19***
Fruit		**PAMi**	213.93/0.40 - 358.19***
		PAMa	217.28/1.09 - 352.04***
FS	3.44/3.00 - 7.00***	**PIA**	0.30/0.00 - 0.87***
FW	98.15/0.54 - 353.67***	***Distal fruit end shape***	
FSH	3.79/1.00 - 5.00***	**DAMi**	140.76/0.55 - 358.33***
FSUR	1.34/1.00 - 3.00***	**DAMa**	116.87/0.28 - 330.55***
FCSC	3.81/1.00 - 5.00***	**DIA**	0.00/0.00 - 0.39*
CAPS	0.20/0.00 - 1.00***	**DEP**	0.04/0.00 - 0.69***
AS	0.07/0.00 - 1.00***	***Asymmetry***	
FSPA	3.75/1.00 - 5.00***	**OB**	0.00/-0.21 - 0.22***
FSBE	2.16/1.00 - 4.00***	**OV**	0.37/0.00 - 0.89***
Lu	50.51/33.00 - 74.89***	**VA**	0.38/0.01 - 1.57***
CHRu	26.49/9.85 - 36.74***	**HAOb**	0.03/0.00 - 1.13***
HUEu	125.60/99.76 - 148.75**	**HAOv**	1.16/0.00 - 6.46***
Lr	41.35/12.49 - 68.76***	**WWP**	0.28/0.04 - 0.67***
CHRr	27.15/11.60 - 45.36***	***Internal eccentricity***	
HUEr	46.06/7.89 - 108.06***	**ECC**	0.64/0.10 - 0.82***
		PE	0.89/0.12 - 1.37***
Agronomic		**DE**	0.86/-4.37 - 2.45*
		FSII	1.96/0.29 - 14.34***
Y	1183.18/127 - 3872***	**EAI**	0.53/0.38 - 0.93***

[a] ***, **, *, ns indicate significant for $P<0.001$, $P<0.01$, $P<0.05$, and non-significant, respectively, obtained after analysis of variance (ANOVA) for individual accession values.

Study of variation between and within varietal groups - *Conventional descriptors*

Considering varietal groups means, significant differences were found for 30 of the 32 studied conventional descriptors (Table 5). CSC and AC showed no significant differences among the nine varietal groups. As explained previously, CSC is not a useful trait to differentiate materials within the *annuum* complex. AC may be a discriminating trait for collections encompassing different morphologies, but not for the varietal groups considered in our collection. Regarding AC, the *annuum* complex usually presents purple anthers but, in some cases, it also shows shades of blue to violet, as in our collection, while yellow or pale anthers are very unusual (DeWitt and Bosland, 1996; Russo, 2012).

For most traits with significant differences among varietal groups, groups fell into different categories, showing a clear separation (Table 5). Fruit traits FW, FSH and FSPA, and agronomic trait Y were able to separate varietal groups into several categories and were therefore the most discriminant. On the other hand, for some traits significant differences among groups were only due to one varietal group that differed from the others. Thus, LD and FP enabled to differentiate group VIII from the other groups. The same happened for CC and group IX, and finally FS for group IV. CAPS separated the first four groups from the others, where some (or even all) accessions were pungent (Table 5).

Intra-group variability was also observed for several conventional descriptors. Based on the standard deviations, descriptors such as NA and LS (plant), CAC (flower), FW, CAPS, AS (fruit), and, as expected, Y (agronomic) were the ones responsible for most of the observed intra-group variation, including *Morrón* types (Table 5). In the case of CAPS and AS, intra-variability was only observed in the more heterogeneous groups (V, VII and IX), where some accessions differed for these traits. The intra-varietal diversity may have an important impact in future pepper breeding programs for these materials by providing the opportunity to select those traits that have a major acceptance by consumers and producers (Costa et al., 2011; Parisi et al., 2017).

Table 5 – Means (± standard deviation) for conventional descriptors corresponding to the varietal groups considered.

Descriptor	Group I (MC)	Group II (MV)	Group III (MB)	Group IV	Group V	Group VI	Group VII	Group VIII	Group IX (CON)
PH	93.95 ± 20.80 a[ǂ]	102.29 ± 22.78 ab	96.85 ± 14.47 ab	97.32 ± 15.00 ab	133.15 ± 31.38 c	113.16 ± 21.70 b	108.27 ± 26.05 ab	140.62 ± 56.46 c	146.19 ± 26.81 c
GH	4.91 ± 0.41 a	5.00 ± 0.00 ab	4.85 ± 0.54 a	5.29 ± 0.71 ab	5.40 ± 0.82 b	5.25 ± 0.67 ab	5.14 ± 0.92 ab	6.00 ± 1.03 c	6.00 ± 1.03 c
NA	4.76 ± 2.41 bc	4.18 ± 2.52 ab	5.15 ± 2.30 bc	4.71 ± 2.29 ab	5.40 ± 2.01 bc	6.00 ± 1.76 c	5.71 ± 1.96 bc	6.00 ± 1.79 c	3.00 ± 2.53 a
SL	20.00 ± 4.27 ab	20.70 ± 3.03 ab	19.88 ± 6.43 ab	22.84 ± 7.06 ab	21.26 ± 5.17 ab	21.53 ± 4.95 ab	18.45 ± 5.60 a	34.27 ± 15.26 c	22.62 ± 3.65 b
BH	4.60 ± 1.08 bc	4.76 ± 1.18 bc	4.08 ± 1.28 ab	4.71 ± 0.71 bc	5.00 ± 0.00 c	5.50 ± 1.68 c	3.86 ± 1.00 a	5.50 ± 1.71 c	5.50 ± 0.89 c
LD	4.89 ± 1.05 ab	4.41 ± 1.35 a	4.69 ± 1.08 ab	5.00 ± 1.09 ab	5.40 ± 0.82 b	4.50 ± 0.88 a	4.57 ± 1.36 a	6.00 ± 1.03 c	5.00 ± 0.00 ab
LS	1.42 ± 0.54 ab	1.71 ± 0.46 bcd	1.62 ± 0.49 bc	1.14 ± 0.36 a	1.40 ± 0.50 ab	2.88 ± 0.34 e	1.93 ± 0.71 cd	2.00 ± 0.73 d	1.25 ± 0.45 a
MLL	16.03 ± 2.59 d	15.98 ± 3.06 d	16.27 ± 2.70 d	16.29 ± 3.53 d	15.17 ± 2.08 d	10.21 ± 2.05 ab	13.90 ± 2.58 c	10.71 ± 2.35 b	9.49 ± 1.27 a
MLW	8.61 ± 1.43 d	8.28 ± 1.73 d	9.03 ± 1.66 d	8.71 ± 2.01 d	8.39 ± 1.41 d	4.32 ± 0.88 a	7.12 ± 1.53 c	5.27 ± 1.20 b	5.42 ± 0.76 b
FA	1.00 ± 0.00 a	1.00 ± 0.00 a	1.08 ± 0.27 a	1.00 ± 0.00 a	1.00 ± 0.00 a	1.25 ± 0.67 b	1.00 ± 0.00 a	1.00 ± 0.00 a	1.75 ± 0.45 c
CC	1.00 ± 0.00 a	1.00 ± 0.00 a	1.00 ± 0.00 a	1.00 ± 0.00 a	1.00 ± 0.00 a	1.00 ± 0.00 a	1.00 ± 0.00 a	1.00 ± 0.00 a	3.25 ± 1.34 b
CSC	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
AC	4.91 ± 0.59 a	5.00 ± 0.00 a	5.08 ± 0.27 a	5.14 ± 0.36 a	5.20 ± 0.41 a	5.00 ± 0.00 a	5.00 ± 0.38 a	5.00 ± 0.00 a	5.00 ± 0.00 a
FP	6.56 ± 1.02 d	7.00 ± 0.00 d	6.69 ± 1.08 d	6.43 ± 1.43 cd	5.40 ± 1.54 b	5.25 ± 1.22 b	5.86 ± 1.47 bc	3.50 ± 0.89 a	7.00 ± 0.00 d
CM	2.87 ± 0.34 cd	3.00 ± 0.00 d	2.77 ± 0.43 cd	2.86 ± 0.36 cd	2.80 ± 0.41 cd	3.00 ± 0.00 d	2.64 ± 0.48 bc	2.50 ± 0.52 b	2.00 ± 0.00 a
CAC	0.91 ± 0.28 d	0.88 ± 0.34 d	0.62 ± 0.49 c	1.00 ± 0.00 d	1.00 ± 0.00 d	0.12 ± 0.34 a	0.36 ± 0.48 b	0.25 ± 0.45 ab	0.75 ± 0.45 cd
FS	3.53 ± 1.22 a	3.00 ± 0.00 a	3.31 ± 0.73 a	4.43 ± 1.79 b	3.80 ± 1.64 a	3.00 ± 0.00 a	3.29 ± 1.04 a	3.00 ± 0.00 a	3.50 ± 0.89 a
FW	142.78 ± 48.8 gh	160.52 ± 46.29 h	95.35 ± 26.27 f	111.44 ± 21.14 fg	41.17 ± 11.14 e	8.10 ± 6.61 b	33.22 ± 19.56 d	12.48 ± 7.21 c	8.01 ± 6.77 a
FSH	5.00 ± 0.00 e	5.00 ± 0.00 e	2.69 ± 1.28 c	3.57 ± 0.92 d	3.00 ± 0.00 d	1.00 ± 0.00 a	3.00 ± 1.87 c	2.00 ± 1.03 b	2.50 ± 1.15 c
FSUR	1.09 ± 0.28 a	1.00 ± 0.00 a	1.00 ± 0.00 a	1.14 ± 0.36 a	1.20 ± 0.41 a	3.00 ± 0.00 c	1.79 ± 0.41 b	1.00 ± 0.00 a	2.00 ± 1.03 b
FCSC	4.67 ± 0.80 d	4.88 ± 0.48 d	2.46 ± 1.09 ab	4.57 ± 0.50 d	3.00 ± 1.72 bc	2.00 ± 0.00 a	3.21 ± 1.84 c	2.00 ± 0.73 a	3.25 ± 1.34 c
CAPS	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.40 ± 0.50 b	1.00 ± 0.00 d	0.36 ± 0.48 b	1.00 ± 0.00 d	0.75 ± 0.45 c
AS	0.11 ± 0.31 ab	0.24 ± 0.43 b	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.25 ± 0.45 b	0.00 ± 0.00 a
FSPA	4.51 ± 0.58 f	4.24 ± 0.43 f	3.38 ± 0.49 d	4.14 ± 0.36 f	3.80 ± 0.77 e	1.00 ± 0.00 a	3.57 ± 0.83 de	2.00 ± 0.73 b	2.50 ± 0.89 c
FSBE	2.76 ± 0.64 c	2.53 ± 0.71 c	2.08 ± 0.27 b	2.00 ± 1.22 b	1.00 ± 0.00 a	1.00 ± 0.00 a	1.79 ± 0.87 b	1.00 ± 0.00 a	1.25 ± 0.45 a
Lu	49.03 ± 5.34 ab	51.36 ± 8.77 ab	47.47 ± 6.58 ab	46.08 ± 4.20 a	50.49 ± 4.90 b	55.68 ± 6.21 c	55.93 ± 6.91 c	54.73 ± 7.34 c	49.72 ± 5.52 ab
CHRu	25.58 ± 3.20 bc	26.16 ± 3.55 bc	23.50 ± 4.44 a	24.83 ± 2.31 b	28.75 ± 2.29 d	29.65 ± 3.63 d	29.19 ± 2.83 d	27.75 ± 3.32 cd	30.22 ± 3.75 d
HUEu	125.30 ± 4.51 ab	125.26 ± 6.98 b	126.24 ± 4.93 b	128.62 ± 3.22 b	125.96 ± 2.98 b	124.92 ± 5.23 ab	125.09 ± 3.55 ab	122.81 ± 5.25 a	127.84 ± 2.59 bc
Lr	42.26 ± 7.87 b	43.93 ± 5.39 b	40.67 ± 5.68 b	38.51 ± 4.83 b	40.59 ± 2.15 b	38.22 ± 4.13 b	43.41 ± 9.20 b	40.30 ± 5.13 b	33.72 ± 6.09 a
CHRr	24.96 ± 4.67 a	24.73 ± 4.90 a	24.75 ± 5.69 a	25.29 ± 5.02 a	28.52 ± 4.56 b	32.98 ± 5.47 c	31.54 ± 3.48 b	30.96 ± 1.55 c	35.54 ± 6.84 c
HUEr	51.54 ± 20.34 b	51.77 ± 10.95 b	45.80 ± 10.90 b	41.74 ± 7.37 b	40.75 ± 4.17 a	32.92 ± 10.37 b	42.82 ± 13.12 b	42.27 ± 7.77 b	28.14 ± 5.35 a
Y	1250.12 ± 525.04 e	1725.94 ± 568.50 cd	1298.46 ± 646.28 cd	1352.96 ± 446.24 d	1123.15 ± 278.35 cd	644.66 ± 262.03 a	982.02 ± 399.41 bc	733.25 ± 470.27 a	902.75 ± 455.85 ab

[ǂ] Different letters indicate significant differences among varietal groups, according to Student-Newman-Keuls post-hoc test for $P<0.05$.

Study of variation between and within varietal groups - *Digital phenotyping*

Significant differences among varietal groups were detected for 30 of the 35 digital traits and parameters. Traits DIA, DEP, OB, HAOb, and DE showed no significant differences among varietal groups.

A considerable diversity was found among varietal groups. Most traits, 29 out of 30, separated the groups into several categories, with the only exception of PE, for which significant variability was only found for group VI. On the whole, digital phenotyping separated varietal groups into more categories than those observed for conventional descriptors, suggesting a higher capability to differentiate among varietal pepper types. Basic descriptors like AR, WMH, MH, and CH were the ones with higher discriminating ability. Even more, digital phenotyping was able to separate morphologically close varietal groups such as *Morrón de Cascos* (group I), *Valenciano* (group II), *Morrón de Bola* (group III), and group IV. Our results are similar to others reported for a collection of tomato landraces, where digital phenotyping detected a higher number of differences among closely related accessions (Figàs et al., 2014).

Furthermore, a considerable intra-group variation, indicated by standard deviation values, was also detected by the digital traits and parameters. Fruit shape index trait FSIE.I, proximal fruit end shape SH, PAMa and PIA, distal end fruit shape DAMi and DAMa, asymmetry VA, HAOv and WWP, and finally internal eccentricity FSII were the parameters associated to higher variance levels within varietal groups. These findings are consistent to others from a recent work encompassing several *Capsicum* species which also reported a remarkable variation for these traits (Tripodi and Greco, 2018). In our work this ability to detect variation has been extended to closely related materials within specific varietal pepper types, which is of paramount importance for registration and typification purposes.

Table 6 – Means (± standard deviation) for digital traits corresponding to the varietal groups considered.

Trait	Group I (MC)	Group II (MV)	Group III (MB)	Group IV	Group V	Group VI	Group VII	Group VIII	Group IX (CON)
P	39.08 ± 6.57 d [1]	44.95 ± 3.86 e	26.39 ± 2.68 c	41.09 ± 5.04 de	27.31 ± 3.23 c	30.20 ± 9.61 c	30.94 ± 7.74 c	15.30 ± 4.10 b	13.43 ± 2.59 a
AR	71.33 ± 18.80 e	90.67 ± 12.19 f	39.82 ± 7.95 d	71.78 ± 15.39 e	36.54 ± 10.23 d	17.31 ± 9.23 c	36.38 ± 16.31 d	12.68 ± 5.18 b	8.82 ± 3.42 a
WMH	8.53 ± 1.72 f	7.32 ± 1.11 f	8.08 ± 0.64 f	6.15 ± 1.12 e	4.65 ± 1.44 d	1.42 ± 0.44 a	3.60 ± 1.07 c	2.46 ± 0.58 b	2.65 ± 1.52 b
MW	9.23 ± 1.55 e	8.78 ± 0.53 e	8.34 ± 0.60 e	7.87 ± 1.18 e	5.66 ± 1.29 c	3.15 ± 1.51 a	4.61 ± 1.00 b	2.73 ± 0.71 a	3.01 ± 1.41 a
HMW	7.93 ± 2.72 c	12.05 ± 2.87 d	5.02 ± 1.15 b	11.73 ± 1.82 c	8.21 ± 0.78 c	9.34 ± 4.89 d	9.98 ± 3.49 cd	5.38 ± 1.45 b	3.87 ± 0.98 a
MH	10.6 ± 2.54 cd	14.39 ± 2.39 f	6.19 ± 1.12 b	13.69 ± 1.78 ef	9.33 ± 0.98 c	12.75 ± 4.11 de	11.54 ± 3.55 d	5.69 ± 1.58 b	4.37 ± 1.21 a
CH	11.92 ± 2.68 e	15.01 ± 1.90 g	6.52 ± 1.20 c	14.19 ± 1.87 fg	9.63 ± 1.18 d	13.47 ± 4.24 ef	11.75 ± 3.50 e	5.79 ± 1.56 b	4.55 ± 1.24 a
FSIE I	1.18 ± 0.36 b	1.65 ± 0.32 c	0.74 ± 0.13 a	1.78 ± 0.34 c	1.73 ± 0.40 c	4.73 ± 2.24 e	2.55 ± 0.75 d	2.12 ± 0.42 d	1.84 ± 0.94 c
FSIE II	1.00 ± 0.50 b	1.76 ± 0.68 c	0.63 ± 0.16 a	1.99 ± 0.51 c	2.01 ± 0.82 c	6.76 ± 3.43 e	2.94 ± 1.15 d	2.23 ± 0.53 de	2.06 ± 1.20 c
CFSI	1.48 ± 0.52 b	2.24 ± 0.73 c	0.81 ± 0.16 a	2.54 ± 0.62 c	2.30 ± 0.87 c	9.77 ± 2.52 e	3.56 ± 1.31 d	2.40 ± 0.50 c	2.46 ± 1.52 c
PFB	0.93 ± 0.18 b	1.03 ± 0.14 bcd	0.82 ± 0.19 a	1.20 ± 0.23 cd	1.17 ± 0.32 bcd	1.12 ± 0.21 bcd	1.23 ± 0.19 d	0.96 ± 0.15 bc	0.99 ± 0.19 bc
DFB	0.64 ± 0.14 cd	0.72 ± 0.10 d	0.61 ± 0.10 bc	0.55 ± 0.10 bc	0.46 ± 0.10 a	0.64 ± 0.21 cd	0.58 ± 0.15 bc	0.56 ± 0.07 bc	0.53 ± 0.11 ab
FST	1.56 ± 0.50 a	1.50 ± 0.36 a	1.42 ± 0.47 a	2.24 ± 0.58 cd	2.59 ± 0.66 d	2.00 ± 0.80 bc	2.23 ± 0.69 cd	1.76 ± 0.40 ab	1.97 ± 0.62 bc
ELL	0.11 ± 0.02 b	0.11 ± 0.01 b	0.08 ± 0.02 a	0.12 ± 0.02 b	0.11 ± 0.02 b	0.17 ± 0.05 c	0.12 ± 0.02 b	0.07 ± 0.01 a	0.08 ± 0.02 a
CIR	0.17 ± 0.04 a	0.21 ± 0.05 b	0.16 ± 0.06 a	0.24 ± 0.05 b	0.21 ± 0.07 b	0.45 ± 0.02 d	0.31 ± 0.08 c	0.24 ± 0.05 b	0.24 ± 0.11 b
RECT	0.55 ± 0.06 c	0.52 ± 0.07 c	0.55 ± 0.04 c	0.43 ± 0.07 b	0.45 ± 0.07 b	0.28 ± 0.10 a	0.44 ± 0.09 b	0.51 ± 0.06 c	0.45 ± 0.09 b
SH	0.10 ± 0.04 e	0.08 ± 0.02 de	0.09 ± 0.04 e	0.07 ± 0.03 cd	0.05 ± 0.03 bc	0.05 ± 0.06 bc	0.06 ± 0.04 cd	0.02 ± 0.01 a	0.03 ± 0.03 ab
PAMi	229.81 ± 32.06 c	246.20 ± 21.71 c	205.26 ± 13.63 bc	240.06 ± 24.74 c	229.03 ± 16.79 c	58.07 ± 29.62 a	235.20 ± 56.82 bc	207.91 ± 49.06 bc	201.10 ± 69.87 b
PAMa	259.53 ± 38.28 d	236.21 ± 50.66 cd	252.92 ± 16.26 d	234.19 ± 32.09 cd	227.09 ± 17.68 cd	32.81 ± 24.48 a	193.18 ± 82.71 c	139.76 ± 62.94 b	162.35 ± 47.94 cd
PIA	0.37 ± 0.20 c	0.36 ± 0.14 c	0.33 ± 0.14 c	0.35 ± 0.20 c	0.22 ± 0.12 b	0.00 ± 0.01 a	0.35 ± 0.23 c	0.08 ± 0.06 a	0.10 ± 0.10 a
DAMi	190.50 ± 87.80 c	160.86 ± 64.37 c	164.33 ± 39.11 c	109.17 ± 29.47 c	86.82 ± 30.51 ab	25.61 ± 14.01 a	101.56 ± 76.00 ab	101.54 ± 29.18 ab	80.96 ± 52.20 b
DAMa	171.88 ± 79.28 c	109.96 ± 53.92 bc	145.85 ± 43.55 c	62.02 ± 22.76 b	80.34 ± 28.31 bc	16.10 ± 9.92 a	60.18 ± 41.43 b	71.98 ± 19.18 bc	86.66 ± 52.75 bc
DIA	0.01 ± 0.04 a	0.00 ± 0.00 a	0.00 ± 0.01 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
DEP	0.04 ± 0.11 a	0.01 ± 0.03 a	0.00 ± 0.02 a	0.05 ± 0.16 a	0.06 ± 0.07 a	0.10 ± 0.18 a	0.09 ± 0.19 a	0.00 ± 0.00 a	0.01 ± 0.03 a
OB	0.00 ± 0.04 a	0.00 ± 0.01 a	0.02 ± 0.05 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
OV	0.30 ± 0.15 ab	0.40 ± 0.08 c	0.23 ± 0.12 a	0.52 ± 0.16 de	0.56 ± 0.13 e	0.44 ± 0.15 cd	0.52 ± 0.17 de	0.37 ± 0.13 bc	0.39 ± 0.13 c
VA	0.44 ± 0.22 cd	0.54 ± 0.29 de	0.23 ± 0.13 ab	0.48 ± 0.38 d	0.21 ± 0.13 ab	0.65 ± 0.41 e	0.31 ± 0.21 bc	0.11 ± 0.13 a	0.15 ± 0.14 ab
HAOb	0.05 ± 0.20 a	0.02 ± 0.08 a	0.03 ± 0.09 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
HAOv	0.98 ± 0.71 b	1.59 ± 0.63 cd	0.43 ± 0.24 a	1.91 ± 0.52 de	1.33 ± 0.45 c	2.21 ± 1.01 e	1.60 ± 0.80 cd	0.49 ± 0.30 a	0.52 ± 0.41 a
WWP	0.32 ± 0.12 d	0.21 ± 0.05 ab	0.40 ± 0.08 e	0.16 ± 0.03 a	0.18 ± 0.08 a	0.23 ± 0.13 abc	0.20 ± 0.11 a	0.29 ± 0.10 cd	0.27 ± 0.10 bcd
ECC	0.59 ± 0.10 ab	0.65 ± 0.07 bc	0.65 ± 0.06 bc	0.68 ± 0.06 c	0.70 ± 0.03 cd	0.56 ± 0.22 a	0.70 ± 0.05 cd	0.77 ± 0.02 d	0.70 ± 0.15 cd
PE	0.90 ± 0.05 b	0.89 ± 0.02 b	0.90 ± 0.03 b	0.89 ± 0.02 b	0.89 ± 0.02 b	0.79 ± 0.34 a	0.89 ± 0.04 b	0.89 ± 0.02 b	0.89 ± 0.02 b
DE	0.87 ± 0.06 a	0.88 ± 0.04 a	0.89 ± 0.01 a	0.88 ± 0.07 a	0.87 ± 0.03 a	0.65 ± 1.25 a	0.90 ± 0.06 a	0.90 ± 0.05 a	0.86 ± 0.15 a
FSII	1.03 ± 0.50 b	1.78 ± 0.68 a	0.64 ± 0.18 a	2.09 ± 0.52 b	2.05 ± 0.83 b	6.70 ± 3.51 c	3.04 ± 1.16 d	2.26 ± 0.52 b	2.00 ± 1.22 b
EAI	0.54 ± 0.07 cd	0.56 ± 0.07 d	0.50 ± 0.04 bc	0.52 ± 0.07 bcd	0.50 ± 0.04 bc	0.61 ± 0.15 e	0.49 ± 0.05 bc	0.44 ± 0.03 a	0.48 ± 0.12 b

[1] Different letters indicate significant differences among groups according to Student-Newman-Keuls post-hoc test for P<0.05.

Pairwise differences among varietal groups

The number of pairwise significantly different traits can illustrate how varietal groups differed from each other depending on the method of phenomics used (Table 7). Thus, considering conventional descriptors, groups VIII and IX encompassed the highest mean number of significantly different traits (19 and 20, respectively) to other groups, whereas the groups corresponding to *Morrón* peppers were the ones with least significant differences (12-13), followed by groups IV and V (13-14) (Table 7). Group IX included four accessions belonging to three different species with several traits that make them unique within the collection. On the other hand, group I included a large cluster of accessions of *Morrón de Cascos* type with several plant, flower, and fruit traits common to other varietal groups. Thus, *Morrón de Cascos*, *Valenciano*, *Morrón de Bola*, and thick fleshed peppers (IV) groups share many traits, and therefore showed a relative low number of pairwise differences among them (ranging from 1 to 8) (Table 7). This indicates a close relationship for these groups which was also confirmed by DNA polymorphisms in a recent work (Pereira-Dias et al., 2019).

By contrast, for digital phenotyping, the number of pairwise significant differences was generally higher in comparison to the conventional traits, particularly for the *Morrón* peppers groups. On the whole, group VI was the one with the highest mean number of significantly different descriptors (23), followed by *Morrón de Bola* (20), while group IV showed the lowest number of pairwise significant differences (15) (Table 7). Also, in contrast to the findings with conventional descriptors, groups VIII and IX (18-19) were not the ones with the highest number of differences, despite considering three different species in the case of group IX (Table 1). In fact, the number of pairwise differences was similar or lower than observed for conventional characterization. As Tomato Analyzer only takes into account fruit traits, these results suggest that the traits that made these groups unique in the conventional characterization were particularly plant traits. Thus, the group IX includes a round shaped *C. annuum* accession (*Pimiento de Bola*) which shares many traits with the *Morrón* groups, especially with round-shaped fruits of group III. The other three accessions of this group are Ají chirere (*C. frutescens*), ECU-994 and Habanero (*C. chinense*), and the first shares many resemblance to a short cayenne or a Serrano (group VIII), the second has triangular shape with pointed end and without shoulders, similar to the groups VI and VIII, and finally the third has irregular round shape and slightly pointy distal end which could be the middle ground between a *Morrón de Bola* and a Jalapeno.

In addition, with similar number of traits and parameters as the conventional characterization, digital phenotyping enabled to detect a higher mean number of differences between varietal groups (Table 7). Thus, as an example, conventional descriptors were only able to detect 1, 8, and 4 significantly different traits between *Morrón de Cascos* and the three closest varietal groups *Valenciano*, *Morrón de Bola*

and thick fleshed peppers from group IV, respectively, whereas considering the same pairwise comparisons digital phenotyping increased to 13, 14, and 16 parameters with significant differences among the mentioned varietal groups (Table 7). Regardless this phenomics tool only analyses fruit parameters, it provides higher discrimination power, essential for germplasm characterization and typification (Figàs et al., 2014; Hurtado et al., 2013).

Table 7 – Number of pairwise significantly different (P<0.05) for varietal groups for conventional descriptors (above the diagonal and highlighted in blue) and for digital traits and parameters (below the diagonal and highlighted in grey). The average number of significant of differences for each group is also provided.

Varietal groups	Group I (MC)	Group II (MV)	Group III (MB)	Group IV	Group V	Group VI	Group VII	Group VIII	Group IX (CON)	Mean of conventional
Group I (MC)		1	8	4	13	18	15	18	21	**12.25**
Group II (MV)	13		8	6	12	19	17	19	21	**12.88**
Group III (MB)	14	17		7	11	17	11	21	21	**13.00**
Group IV	16	8	21		12	19	16	23	21	**13.50**
Group V	20	17	21	11		17	13	20	16	**14.25**
Group VI	25	24	28	21	25		16	15	19	**17.50**
Group VII	19	18	21	12	10	19		18	22	**16.00**
Group VIII	22	21	19	21	20	21	22		21	**19.38**
Group IX (CON)	24	19	20	16	14	23	19	10		**20.25**
Mean of digital	**19.13**	**17.13**	**20.13**	**15.75**	**17.25**	**23.25**	**17.50**	**19.50**	**18.13**	

Principal components analysis (PCA) - *Conventional descriptors*

Only conventional descriptors which provided significant differences: i) among accessions considering the whole collection and ii) among varietal groups were considered to perform PCA. The first two principal components explained 35.89% of total variance (Figure 2). PC_1 explained 27.03% of total variance and was positively correlated with fruit traits CAPS, FSUR and CHRr, and negatively to fruit traits FW, FSPA, FSH and plant traits MLW and MLL (Table 8). In addition, PC_2 accounted for 8.87% of variation and was positively correlated with fruit traits Lu and CHRu, and to plant descriptors LS and NA, whereas flower descriptors CC and FA, fruit descriptor HUEu, and plant trait PH were negatively correlated with PC_2 (Figure 2). Therefore, fruit traits were responsible for most of the explained variance (Table 8).

As a result, pungent, wrinkled and less saturated (lighter) red colour fruits like the ones included in group VI and some accessions of groups VIII and IX appeared in the positive side of PC_1 (Figure 2). By contrast, most accessions with big fruits, lobate pedicel attachment, and big leaves, such as the *Morrón* groups and fleshy peppers (groups I to IV), appeared in the negative side of PC_1. Likewise, accessions with lighter colour immature fruits, ovate or lanceolate leaves, and higher content of anthocyanin in the nodes were located on the top while accessions with yellow-greenish corollas, two flowers per axil, dark green immature fruits and taller plants were placed on the bottom of the graph (Figure 2).

The use of standardized descriptors is an important practice for germplasm identification (Bioversity International, 2017; Gotor et al., 2008; UPOV, 2019). However, its discrimination power is sometimes limited, especially when differences among materials are very subtle fruit traits (Brewer et al., 2006; Costa et al., 2011; Figàs et al., 2014). The set of 31 conventional descriptors was able to separate clearly distinct materials such as group VI and the *C. chinense* and *C. frutescens* accessions. In the same way, in another report, for a remarkably diverse collection encompassing nine species, leaf shape, nodal anthocyanin, and several flower traits were the most informative descriptors, indicating their usefulness when working with interspecific collections (Tripodi and Greco, 2018). Unfortunately, for closely related materials, particularly the ones included in our collection, separation was not so satisfactory (Figure 2).

Thus, fruit traits explained the highest percentage of variance for this collection. However, conventional descriptors lack of detail to be fully descriptive of the subtle differences between these accessions. In this regard, other works also reported an insufficient resolution of conventional descriptors and that fruit traits explain most of the variance (Costa et al., 2011; Figàs et al., 2014; Hurtado et al., 2013). Thus, as found in other crops, pepper varietal types are displayed mainly based on fruit shape, colour and flesh culinary properties (i.e. to eat fresh, fried, dry, roasted, as dip, etc.) which is

in agreement with the fact that fruit traits explain a higher percentage of the variability (Bosland and Votava, 2012; Rivera et al., 2016; Tripodi and Greco, 2018).

Principal components analysis (PCA) - *Digital phenotyping*

Based on Tomato Analyzer traits and parameters the first two principal components explained 53.17% of total variance for our collection, 35.29 and 17.88% corresponding to PC_1 and PC_2, respectively, which was considerably higher than those recorded with convention descriptors. PC_1 was positively correlated with fruit shape index descriptors CFSI, FSIE.I, FSIE.II, internal eccentricity descriptor FSII, and homogeneity descriptor CIR, while on the other hand, it was negatively correlated with homogeneity descriptor RECT, basic descriptors WMH and MW, proximal end shape descriptor PAMa, and distal end shape descriptor DAMa (Table 8). PC_2 was positively correlated with basic descriptors P, CH, MH, AR, HMW and MW, while it was negatively correlated to asymmetry descriptor WWP (Table 8).

Thus, accessions bearing fruits with high height/width ratios, between internal eccentricity and width, were located in the positive side of PC_1, whereas wider and rectangular fruits and wider proximal and distal ends appeared on the negative side (Figure 2). In this way, accessions with elongated shape, as the cayenne from group VI, were on the opposite side to blocky and fleshy peppers from *Morrón* groups I to III and group IV. In addition, PC_2 separated those accessions with bigger fruit sizes, height and width to the upper part of the PCA, while those accessions with higher ratio between height at maximum width and maximum height appeared mainly on the bottom of the PCA (Figure 2).

Despite considering only fruit traits and parameters, digital phenotyping provided a good separation among accessions. A more detailed insight into the PCA clusters shows a better separation of accessions than that observed with conventional descriptors (Figure 2). Varietal groups were discriminated into several sub-clusters, e.g. three sub-groups of different cayenne size appeared from group VI. Likewise group VII accessions were divided into roughly three sub-clusters based on fruit shape. In addition, the phenomics tool was close to separate in the PCA groups I to IV, particularly *Valenciano* from *Morrón de Bola*. This four groups share many characteristics for both plant and fruit, resulting almost impossible to separate them based on conventional descriptors. Digital phenotyping demonstrated a higher accuracy to identify subtle differences in pepper fruits and to separate morphologically close materials, which is in agreement with the higher contribution to variation found for PC_1 and PC_2 with this tool (Figure 2). In other words, our results suggest that the higher percentage of variance detected by digital phenotyping enabled a better separation of the materials along both coordinates, indicating a more efficient differentiation of phenotypes based on a lower

number of scored traits. Our results are in agreement with other works in tomato, where digital phenotyping explained a higher percentage of variability than conventional descriptors and therefore that fruit morphology is essential to assess variation among cultivars and to varietal typification (Figàs et al., 2014).

Nonetheless, not all digital traits contribute similarly to differentiate accessions or varietal groups, as it is highly dependent on the accessions considered. However, there are a set of descriptors that, regardless of the considered collection, explain a high level of diversity. According to our findings, basic and fruit shape index descriptors are usually the most informative, a pattern also reported in several other species (Figàs et al., 2014; Hurtado et al., 2013).

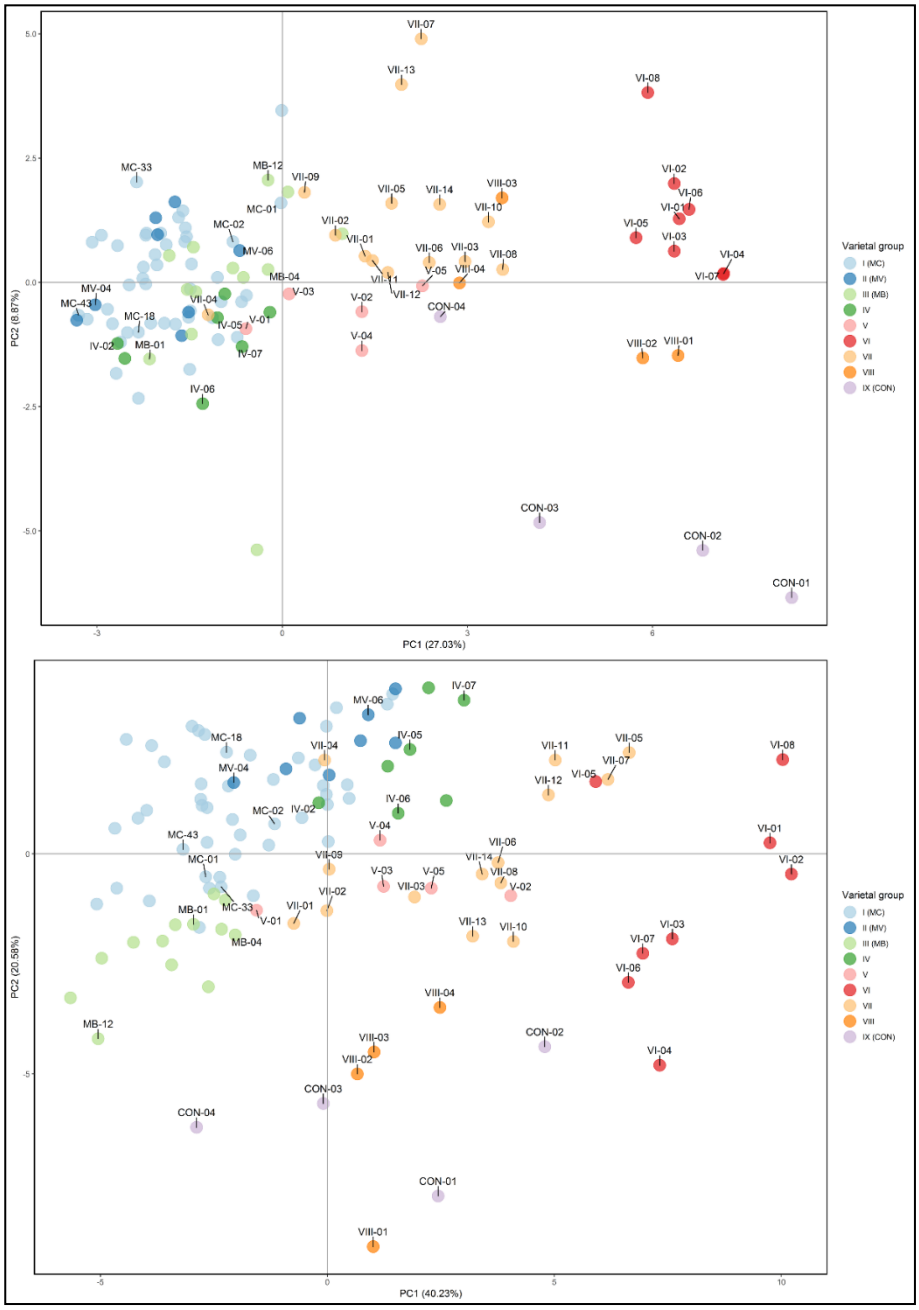

Figure 2 – First and second principal components for the studied pepper accessions using selected conventional descriptors (31; top) and digital traits and parameters (35; bottom). To avoid plot saturation, labels were displayed for accessions from groups V to IX and for only a few representatives from groups I to IV for orientation (i.e. Figure 1 fruits).

PCA combining conventional descriptors and digital phenotyping

After the comparison of both types of descriptors, we proceeded to check how they would behave when considered together. For this, non-significant descriptors were discarded. Thus, 66 traits and descriptors (31 conventional and 35 digital) were considered. The PCA explained 41.02% of total variance in our collection (Figure 3). PC_1 explained 28.34% of variance and was positively correlated with Tomato Analyzer fruit shape index descriptors CFSI, FSIE.I, FSIE.II and internal eccentricity descriptor FSII. Also, it was negatively correlated with Tomato Analyzer basic traits WMH and MW, and with conventional fruit descriptor FW (Table 8). PC_2 accounted for 12.68% of total variance and was positively correlated with Tomato Analyzer basic descriptors MH, CH, HMW, P, AR, and asymmetry descriptor HAOv, while it was negatively correlated to Tomato Analyzer asymmetry descriptor WWP and to IPGRI inflorescence/flower descriptors CC and FA (Table 8).

This enabled the separation of accessions with higher height/width ratio (elongated and thinner fruits) to the positive side of the first component from those accessions with bigger and heavier fruits that located to the left side of the axis. Thus, *cayenne*/*guindilla* and the blocky/*Morrón de Cascos* types appeared in opposite sides of the axis (Figure 3). PC_2 grouped tall, bulky, and accessions with larger proximal area than distal area at the top of the component axis, whereas accessions with higher height at maximum width/total height ratio and non-white corolla and more than one flower per axil were located at the bottom of the plot (Figure 3).

The combination of both sets of traits and descriptors explained an intermediate level of variation than that considering these sets separately, it also provides a detailed separation of accessions and corresponding varietal groups (Figure 3). In any case, the use of both sets of descriptors is admissible in order to get as many morphological differences as possible. In this study, *Morrón de Cascos* group was not separated into a clearly distinct cluster but into a continuum that connects the group III to the groups II and IV. This indicates a relatively wide range of morphologies within this varietal group, where some accessions are closer to the bell peppers with round shape from group II, some others with intermediate form, and the rest with close resemblance to the big, rectangular and triangular, thick fleshed peppers from *Morrón Valenciano* and group IV (Figure 3). Such findings indicate a considerable intra-varietal diversity within *Morrón* peppers, similarly to the reports from other varietal types (Parisi et al., 2017; Rivera et al., 2016).

For groups Ancho/Piquillo and Numex/Padrón peppers (groups V and VII), accessions were distributed along both axis (Figure 3). Thus, group VII accessions 5, 7, 11, and 12 with similar horn shape clustered together. Accessions from group VI formed two clusters by fruit size. On one hand, accessions VI-1, 2, 5, and 8 formed one cluster, while

accessions 3, 4, 6, and 7 formed another. Group VIII Jalapeno peppers clustered relatively near, although variation among them accessions was detected. Accessions from group IX were very different among them but they shared the reduced size fruits so they positioned in the same quadrant of the plot and closer to the most similar fruits. Finally, in the middle of the plot, remaining accessions from groups V and VII clustered together (Figure 3). These accessions present a triangular or slightly triangular shape, with little to no shoulders and roughly the same size.

Figure 3 – First and second principal components for the studied pepper accessions using selected conventional descriptors and digital traits and parameters. To avoid plot saturation, labels were displayed for accessions from groups V to IX and for only a few representatives from groups I to IV for orientation (i.e. Figure 1 fruits).

Our findings indicate a good performance of both methods when used together. This was already tested by other authors with good results (Figàs et al., 2014; Tripodi and Greco, 2018), although it has been now implemented in Spanish pepper landraces, including both very different varietal types and closely related materials sharing similar morphological traits. In addition, we report here a considerable amount of diversity within *C. annuum* and especially within blocky peppers for several morphological and agronomic traits. The exploitation of these resources in future pepper breeding programmes in collaboration with farmers and local communities could translate into the development of highly adapted and highly productive varieties that correspond to the consumer demand (Egea-Fernández et al., 2018; Hurtado et al., 2014; Parisi et al., 2017; Zonneveld et al., 2015).

Table 8 - Correlation coefficients between significant conventional descriptors and digital traits and the first two principal components when used separately and in combination.

		Conventional		Digital		Combination	
Trait	**Type**	**PC_1**	**PC_2**	**PC_1**	**PC_2**	**PC_1**	**PC_2**
PH	Plant	0.16	-0.25			0.09	-0.08
GH	Plant	0.12	-0.21			0.07	-0.06
NA	Plant	0.05	0.23			0.04	0.06
SL	Plant	0.06	-0.16			0.03	-0.04
BH	Plant	0.07	-0.18			0.04	-0.02
LD	Plant	-0.01	-0.24			-0.01	-0.03
LS	Plant	0.15	0.28			0.11	0.04
MLL	Plant	-0.26	-0.05			-0.15	0.11
MLW	Plant	-0.27	-0.10			-0.17	0.07
FA	Infl./flower	0.14	-0.30			0.07	-0.13
CC	Infl./flower	0.13	-0.34			0.06	-0.14
AC	Infl./flower	0.01	0.00			0.00	-0.03
FP	Infl./flower	-0.16	0.09			-0.10	0.07
CM	Infl./flower	-0.08	0.21			-0.04	0.13
CAC	Infl./flower	-0.21	-0.14			-0.12	0.06
FS	Fruit	-0.05	-0.05			-0.04	-0.04
FW	Fruit	-0.32	0.05			-0.20	0.14
FSH	Fruit	-0.26	-0.04			-0.17	0.05
FSUR	Fruit	0.25	0.09			0.18	0.03
FCSC	Fruit	-0.20	-0.09			-0.13	0.08
CAPS	Fruit	0.29	-0.04			0.18	-0.09
AS	Fruit	-0.03	-0.01			-0.02	0.03
FSPA	Fruit	-0.30	-0.04			-0.19	0.08
FSBE	Fruit	-0.24	0.07			-0.17	0.02
Lu	Fruit	0.12	0.35			0.08	-0.01
CHRu	Fruit	0.16	0.23			0.10	0.00
HUEu	Fruit	-0.03	-0.28			-0.01	0.02
Lr	Fruit	-0.09	0.22			-0.05	0.03
CHRr	Fruit	0.21	0.07			0.12	-0.07
HUEr	Fruit	-0.18	0.12			-0.12	0.04
Y	Agronomic	-0.20	0.01			-0.11	0.11
P	Basic			-0.03	0.39	-0.11	0.29
AR	Basic			-0.12	0.34	-0.17	0.22
WMH	Basic			-0.24	0.19	-0.22	0.04
MW	Basic			-0.21	0.25	-0.21	0.11
HMW	Basic			0.13	0.29	0.02	0.30
MH	Basic			0.11	0.35	0.01	0.33
CH	Basic			0.09	0.37	-0.01	0.33
FSIE.I	Fruit shape index			0.27	0.02	0.20	0.14
FSIE.II	Fruit shape index			0.27	0.01	0.20	0.13
CFSI	Fruit shape index			0.27	0.04	0.20	0.16
PFB	Blockiness			0.18	0.08	0.10	0.15
DFB	Blockiness			-0.06	0.04	-0.05	0.00
FST	Blockiness			0.17	0.04	0.11	0.12

Table 8 (continuation)- Correlation coefficients between significant conventional descriptors and digital traits and the first two principal components when used separately and in combination.

		Conventional		Digital		Combination	
Trait	**Type**	**PC_1**	**PC_2**	**PC_1**	**PC_2**	**PC_1**	**PC_2**
ELL	Homogeneity			0.13	0.19	0.07	0.19
CIR	Homogeneity			0.24	-0.02	0.19	0.09
RECT	Homogeneity			-0.25	0.00	-0.18	-0.11
SH	Prox. fruit end shape			-0.17	0.17	-0.15	0.04
PAMi	Prox. fruit end shape			-0.15	0.10	-0.15	0.01
PAMa	Prox. fruit end shape			-0.21	0.10	-0.18	-0.02
PIA	Prox. fruit end shape			-0.15	0.19	-0.15	0.07
DAMi	Dist. fruit end shape			-0.18	0.05	-0.15	-0.03
DAMa	Dist. fruit end shape			-0.21	0.03	-0.17	-0.07
DIA	Dist. fruit end shape			-0.06	-0.02	-0.04	-0.04
DEP	Dist. fruit end shape			0.10	0.08	0.05	0.11
OB	Asymmetry			-0.07	-0.07	-0.04	-0.08
OV	Asymmetry			0.20	0.08	0.12	0.16
VA	Asymmetry			0.05	0.23	0.00	0.19
HAOb	Asymmetry			-0.07	0.07	-0.06	0.01
HAOv	Asymmetry			0.21	0.21	0.11	0.26
WWP	Asymmetry			-0.19	-0.13	-0.10	-0.19
ECC	Internal eccentricity			0.08	-0.09	0.06	-0.02
PE	Internal eccentricity			-0.08	0.07	-0.08	0.01
DE	Internal eccentricity			-0.04	0.00	-0.04	-0.01
FSII	Internal eccentricity			0.26	0.00	0.20	0.12
EAI	Internal eccentricity			0.01	0.12	0.00	0.08

Identification of highly discriminating descriptors

The classification of germplasm based on morphological standardized descriptors is a well-extended practice. However, it is a tedious and time-consuming task that requires a minimum level of expertise and well-defined descriptors. Even when these conditions are met, the classification and differentiation among materials may be compromised due to the close relationship among materials and to the limited ability of most descriptors to find differences (Brewer et al., 2006; Costa et al., 2011; Figàs et al., 2014). Herein, we report the utility of combining conventional and digital descriptors (66 in total) in order to successfully discriminate closely related materials. Notwithstanding, not all descriptors and parameters significantly contributed to the differentiation of the materials so it is pertinent to select a reduced subset of descriptors in order to capture the maximum diversity while reducing the data collecting labour.

Our collection included a comprehensive representation of landraces and heirlooms from the relevant Spanish centre of diversity as well as other peppers from different countries and three different species (Table 1). Most of these materials were closely related and presented similar morphological traits for plant, flower and fruit (Table 7) (Pereira-Dias et al., 2019). We observed that with just 17 most descriptive descriptors we could explain 81.81% of total variability while successfully discriminating the closely related groups *Morrón de Cascos*, *Valenciano*, *Morrón de Bola*, and thick-fleshed peppers of the group IV, as well as the rest of groups, without losing discrimination power (Figure 4). For this we used four conventional descriptors for flower (2) and fruit (2) (FA, CC, FW, and CAPS, respectively) and 13 Tomato Analyzer fruit traits corresponding to basic measurements (7), fruit shape index (3), asymmetry (2), and internal eccentricity (1) (P, AR, WMH, MW, HMW, H, CH, FSIE.I, FSIE.II, CFSI, HAOv, WWP, and FSII, respectively).

As mentioned before, fruit traits explain the majority of the variance for our collection. This is probably linked to the fact that pepper varietal types are set based mainly on fruit shape, colour and culinary uses (Bosland and Votava, 2012; Rivera et al., 2016; Tripodi and Greco, 2018).

Figure 4 - First and second principal components for studied pepper accessions using 17 most discriminating descriptors and traits of both conventional (4) and digital phenotyping (13) methodologies. PCA explained 81.81% of total variance with a similar level of detail then when considering 66 descriptors and traits. To avoid plot saturation, labels were displayed for accessions from groups V to IX and for only a few representatives from groups I to IV for orientation (i.e. Figure 1 fruits).

Conclusions

Thanks to an in-depth characterization based in 67 conventional and digital descriptors and parameters, we have found a considerable inter and intra-varietal variation in the most valued peppers of the Spanish centre of diversity. This characterization has also enabled the identification of a reduced set of descriptors and parameters which can accurately separate varietal groups, as well as accessions within varietal types, even when considering closely related cultivars. Finally, digital phenotyping of fruits based on Tomato Analyzer software results as a fast and efficient tool to complement varietal characterization and typification of *C. annuum* peppers. These findings will be very useful to farmers and breeders devoted to breeding and recovery of heirloom peppers and will boost germplasm characterization and management in seed banks.

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Chapter II: Molecular characterization of *Capsicum* spp.

Use of molecular markers to assist the development of inbred lines under open field conditions: the case of criollo peppers (*Capsicum annuum* L.) from Mexico

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Citation: Pereira-Dias et al. 2017. Use of molecular markers to assist the development of inbred lines under open field conditions: the case of criollo peppers (*Capsicum annuum* L.) from Mexico. *Notulae Botanicae Horti Agrobotanici* 45(2):365-368. DOI:10.15835/nbha45210940

Abstract

Chile peppers are one of the most important crops in Mexico and a plethora of ecotypes can be found there. Most of them are ancient open-pollinated (OP) landraces selected by farmers for uniform phenotype but with an inherent level of genetic diversity, called *criollos*. In this work 15 pepper accessions, encompassing 2 *criollo* lines, their open-pollinated progenies, and 5 controls, were characterized with a set of 36 IPGRI descriptors and 23 SSR markers to assess the effect of open pollination in the inbreeding process. Heterozygosity levels were comprised between 12 and 47% in the progenies, which were similar or lower than those values from parent plants and similar or higher than control cultivars. Also, both progenies and parents showed similar levels of agronomic and morphological uniformity. Our results suggest that this OP program is efficient in terms of reaching enough agronomic uniformity in *criollo* Ancho peppers while preserving certain genetic diversity to confer adaptation to climate change.

Introduction

Peppers (*Capsicum* spp.) are one of the most important vegetables in the world, encompassing a worldwide production of thirty-six million t (FAO, 2014). *Capsicum annuum* is the most diverse and commonly cultivated. Since Mexico is the primary diversity centre of this species, an extraordinary range of varietal types and cultivars can be found there (DeWitt and Bosland, 1996; Kraft, 2009).

Some of the breeding efforts directed to this species in this country has been towards the improvement of valuable landraces and heirlooms (Kraft, 2009). Mexican institutions labour has been improving the *criollo* peppers like the Ancho type, an ancient open-pollinated (OP) landrace that may be a strong candidate as pre-breeding material or as diverse population with higher adaptation/resilience to climate change (Aguilar-Meléndez et al., 2009; Votava et al., 2005). The governmental initiative consists in participatory breeding programs geared towards getting farmers involved for identification of the best individuals from their fields. Then open pollination seeds from these individuals are harvested for the next generation. This approach provides a reasonable balance between both inbreeding and preserving the essential level of diversity for the landrace identity itself (Kraft, 2009).

The goal of this experiment was to assess phenotypic and genotypic diversity within *criollo* peppers as well as allele fixation levels as a result of this traditional plant breeding method.

Material and Methods

Plant material

Two accessions of OP 'Chile Ancho' landrace populations (A and B), grown by farmers in different locations of the Aguascalientes State, their corresponding progenies (a1, a2, a3, a4 and b1, b2, b3, b4), and a set of five control varieties – three OP landraces (C1: 'Serrano', C2: 'Puya', C3: 'Pasilla') and two relatively modern cultivars (M1: 'Paprika' and M2: 'Chile Hungaro') were included in this experiment (Figure 1). Thus, plant materials were provided by reasearchers of the chile pepper breeding program of the Universidad Autónoma de Aguascalientes (UAA, Mexico). Progenies were obtained from four individual plants, selected by farmers and UAA reasearchers in the field trials of types A and B (i.e. plants a1, a2, a3, a4 and b1, b2, b3, b4) and their seeds were obtained separately from open-pollination fruits (i.e. traditional breeding of *criollo* peppers), while parent A and B seeds came from the same original stock than that sown

in the field trials for selections. Seeds were sent to UPV in the frame of a Material Transfer Agreement (MAT).

Figure 1 - Illustration of fruit type for each parental line, progeny, and controls. From top to bottom, left to right: A, a1-4; B, b1-4; C1-3, M1-2.

Experimental procedures

Eight plants per accession were characterized according to thirty-six IPGRI plant, inflorescence, fruit, and seed descriptors (IPGRI, 1995) (Table 1). ANOVA and Principal Components Analysis (PCA) were calculated to assess the differences among individuals using Statgraphics Centurion XVI (StatPoint Technologies, Inc.).
Subsequently, DNA was extracted using modified CTAB method (Doyle and Doyle, 1990) and a pool of the eight plants DNA made for each accession. A twenty-three Single Sequence Repeat (SSR) marker collection (Minamiyama et al., 2006; Nagy et al., 2007; Portis et al., 2007; Yi et al., 2006), enriched with M13 tail and a fluorescent dye (Schuelke, 2000), was used to genotype the collection throughout capillary electrophoresis and ABI PRISM® 3100-Avant (Applied Biosystems, USA) (Table 2). Genetic parameters, such as Heterozygosity (H), Polymorphic Information Content (PIC), Principal Coordinates Analysis (PCoA), and distance matrix were calculated using GenAlex 6.5 (Peakall and Smouse, 2006) and PowerMarker 3.25 (Liu and Muse, 2005).

Table 1 - List of conventional IPGRI (1995) descriptors measured and corresponding units/scale.

Descriptor	Descriptor's units/scale
Plant descriptors	
Plant height	cm
Growth habit	3=Prostrate, 5=Intermediate (compact), 7=Erect
Nodal anthocyanin	1=Green, 3=Light purple, 5=Purple, 7=Dark purple
Stem length	cm
Stem pubescence	3=Sparse, 5=Intermediate, 7=Dense
Branching habit	3=Sparse, 5=Intermediate, 7=Dense
Leaf density	3=Sparse, 5=Intermediate, 7=Dense
Leaf shape	1=Deltoid, 2=Ovate, 3=Lanceolate
Lamina margin	1=Entire, 2=Undulate, 3=Ciliate
Leaf pubescence	3=Sparse, 5=Intermediate, 7=Dense
Mature leaf length	cm
Mature leaf width	cm
Inflorescence/Flower descriptors	
Number of flowers per axil	1=One, 2=Two, 3=Three or more, 4=Many in bunches, each in individual axil
Corolla colour	1=White, 2=Light yellow, 3=Yellow, 4=yellow-green
Corolla spot colour	0=Absent, 1=White, 2=Yellow, 3=Green yellow, 4=Green, 5=purple
Anther colour	1=White, 2=Yellow, 3=Light blue, 4=Blue, 5=Purple, 6=Dark purple
Flower position	3=Pendant, 5=Intermediate, 7=Erect
Calyx margin	1=Entire, 2=Intermediate, 3=Dentate
Calyx annular constriction	0=Absent, 1=Present
Fruit descriptors	
Fruit set	3=Low, 5=Intermediate, 7=High
Fruit weight	g
Fruit shape	1=Elongate, 2=Almost round, 3=Triangular, 4=Campanulate, 5=Blocky
Fruit surface	1=Smooth, 2=semiwrinkled, 3=Wrinkled
Fruit cross-sectional shape	1=Eliptic, 2=Rounded, 3=Quadrangular, 4=Triangular, 5=Irregular
Ripe fruit pungency (tasting)	0=Absent, 1=Present
Anthocyanin spots or stripes	0=Absent, 1=Present
Fruit shape at pedicel attachment	1=Acute, 2=Obtuse, 3=Truncate, 4=Cordate, 5=Lobate
Fruit shape at blossom end	1=Pointed, 2=Blunt, 3=Slunken, 4=Sunken and pointed
Exterior fruit colour lightness (unripe)	0=Black to 100=White
Exterior fruit colour Chroma (unripe)	0=completely unsaturated to 100=fully saturated
Exterior fruit colour HUE (unripe)	0°=red, 90°=yellow, 180°=green, 270°=blue
Exterior fruit colour lightness (ripe)	0=Black to 100=White
Exterior fruit colour Chroma (ripe)	0=completely unsaturated to 100=fully saturated
Exterior fruit colour HUE (ripe)	0°=red, 90°=yellow, 180°=green, 270°=blue
Seed descriptors	
Seed colour	1=Straw, 2=Brown, 3=Black
Seed surface	1=Smooth, 2=Rough, 3=Wrinkled
Seed weight	g

Table 2 - List of 23 SSR markers applied in this work and corresponding bibliographic reference, sequence motif, expected amplicon size (bp), linkage group, number of expected alelles, and expected Polymorphic Information Content (PIC).

Primer	Motif	Expected amplicon size (bp)	Linkage group	Allele number	PIC
CAMS142†	(TA)3...(AC)7... or (AC)12A(TA)8	241	7	5	0.78
CAMS194†	(TA)7...(TG)11AA(TG)3	245	1	3	0.45
CAMS215†	(TA)7(TG)10TC(TG)5T(TG)5	220	7	3	0.52
CAMS336†	(TC)16	157	3	4	0.61
CAMS460†	(TC)20	215	7	3	0.65
CAMS644†	(TG)3...(AG)26	206	4	3	0.61
CAMS806†	(AGA)19	227	10	5	0.79
EPMS426‡	(AT)15	108-118	7	15	0.62
EPMS643‡	(CT)17	195-223	-	9	0.82
EPMS670‡	(AGA)5	107-110	-	2	0.24
EPMS725‡	(TCT)8	142-157	2	5	0.73
EPMS747‡	(TTCT)5	265-285	-	4	0.69
EPMS755‡	(A)12...(T)11	141-150	-	4	0.68
EPMS924‡	(CT)6...(TA)9...(GTA)5	260-295	-	8	0.82
GPMS159☖	(TAA)20	281-317	-	5	0.61
HpmsE010§	(C)12	198	3	-	-
HpmsE015§	(GCA)8	146	5	4	0.52
HpmsE046§	(TC)8	277	11	4	0.49
HpmsE078§	(GAG)6	203	6	-	-
HpmsE111§	(CT)7	219	4	-	-
HpmsE116§	(CTT)12	189	5	9	0.82
HpmsE117§	(CA)6	198	9	-	-
HpmsE123§	(TTTC)6	180	2	-	-

† Minamiyama et al. (2006), ‡ Portis et al. (2006), ☖ Nagy et al. (2007), § Yi et al. (2006).

Results and Discussion

Phenotypic characterization

From the 36 descriptors, only 15 showed significant variation among our collection, which were used to calculate the first two principal components (PC). PC1 and PC2 explained 39.8 and 19.0% of the collection total variability, respectively. For PC1 plant, flower, fruit and seed traits were the most discriminative (stem length, seed weight, fruit weight, fruit shape, fruit cross-sectional corrugation, and anther colour). PC2 showed a similar behaviour where plant and fruit traits accounted for higher discriminatory values (fruit surface, fruit shape at blossom end, and nodal anthocyanin) (Figure 2a) as reported by Pereira-Dias *et al.* (2015) in a collection of Spanish peppers. Based on that, the 2 OP lines and their progenies were clustered together on the right side of the graph (triangular, larger fruits), as it was expected, even though there is no clear separation of the two families, indicating that standard phenotype is maintained despite the lack of controlled pollination. In the middle, the elongated, medium size fruits with a wrinkled

surface, and to the left the smaller fruits with cayenne forms, clearly separated from each other and the rest of clusters (Figure 2b).

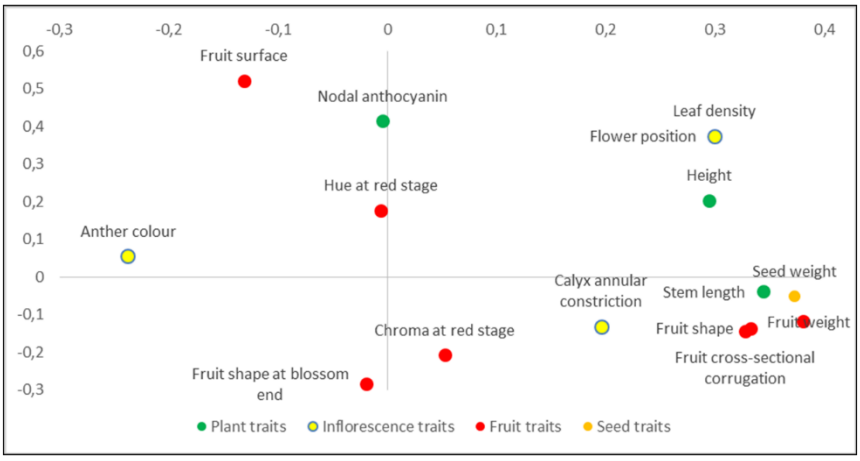

Figure 2a - PC analysis for the first two components and traits distributions based on IPGRI descriptors for Plant (green), inflorescence (yellow), fruit (red), and seed (orange).

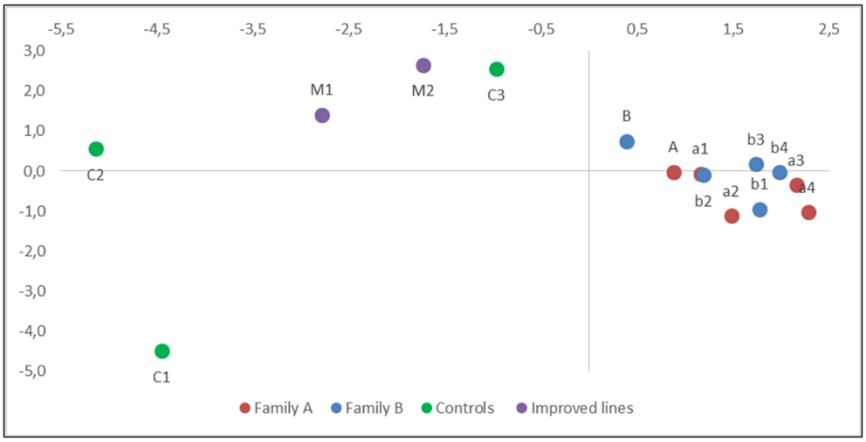

Figure 2b - PC analysis for the first two components and accessions distributions based on IPGRI descriptors. A, B: parental lines; a1-4, b1-4: progenies; C1-3: OP landraces controls; M1-2: modern cultivars controls

Molecular analysis

Six SSR markers were not reliably distinguishable and therefore excluded from the analysis. The remaining 17 SSR allowed the identification of 43 different alleles, with an average of 2.5 alleles per SSR marker, ranging between 2 and 5. PIC mean value was 0.30 (ranging from 0.19 to 0.41). Our results are in agreement with those from Lee *et al.* (2003), Portis *et al.* (2006) and Nagy *et al.* (2007), although PIC mean value was slightly lower, perhaps due to individual's relatedness (Lee et al., 2004; Nagy et al., 2007; Portis et al., 2007).

Observed mean heterozygosity (Ho) was 30% while mean expected heterozygosity (He) was 37%. Ho is higher than expected for an autogamous species. This result might be explained by two factors: i) DNA of eight plants per variety was pooled and ii) open-pollination conditions favoured cross-pollinations. In addition, He value may be that high because of the great number of alleles present. Progenies A and B averaged similar or lower heterozygisity than their corresponding parental lines (Table 3).

Table 3 – Mean observed heterozygosity (Ho) values per accession and progenies.

Family A	Ho	Family B	Ho	Controls	Ho
A	0.29	B	0.41	C1	0.47
a1	0.29	b1	0.29	C2	0.12
a2	0.29	b2	0.47	C3	0.12
a3	0.18	b3	0.12	M1	0.41
a4	0.29	b4	0.35	M2	0.12
μ progenies	0.26		0.31		

A, B: parental lines; a1-4, b1-4: progenies; C1-3: OP landraces controls; M1-2: modern cultivars.

Phylogenetic relationships

Based on the genetic distance matrix a dendrogram was constructed (Figure 3). Clearly, there are two main groups, one corresponding to family A (red) and the other one to family B (blue). Our results suggest that both families show enough genetic differences to be separated despite being closely related phenotypically. Even within families, different levels of genetic fixation are found among progenies and thus, some of them appear closer to the parental line than others.

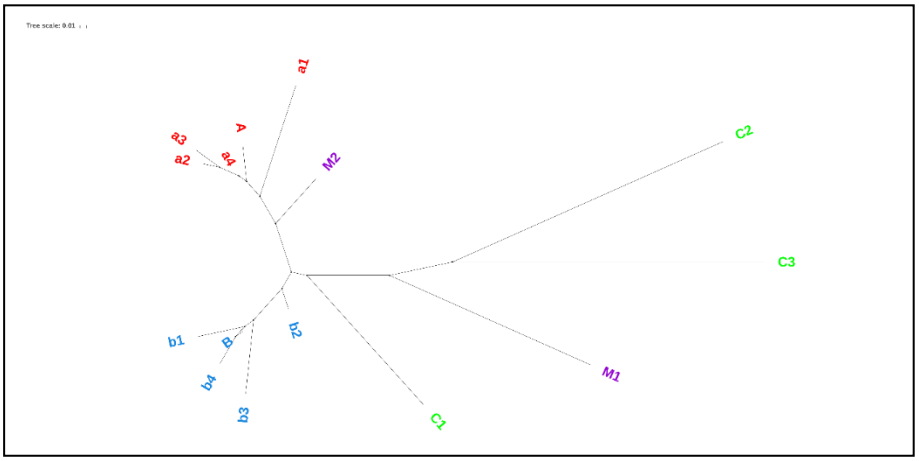

Figure 3 - iTOL's dendrogram (Letunic and Bork, 2016) based on Nei´s genetic distances (Nei et al., 1983). A, B: parental lines; a1-4, b1-4: progenies; C1-3: OP landraces controls; M1-2: modern cultivars controls.

Conclusions

For Chile Ancho breeding a combination between individual selection with open-pollination conditions proved to be a low-cost method that allows: i) the improvement of lines while as well as retaining ii) the expected morphotype inherent to the variety and iii) a certain degree of genetic diversity. The fact that progeny plants had a relatively high level of heterozygosity while being agronomically and morphologically uniform, may provide these materials resilience and adaptation to environmental stress factors and climate change.

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Genetic diversity, population structure and relationships in a collection of pepper (*Capsicum* spp.) landraces from the Spanish centre of diversity revealed by genotyping-by-sequencing (GBS)

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Citation: Pereira-Dias et al. 2019. Genetic diversity, population structure and relationships in a collection of pepper (Capsicum spp.) landraces from the Spanish centre of diversity revealed by genotyping-by-sequencing (GBS). *Horticulture Research* 6:54 https://doi.org/10.1038/s41438-019-0132-8

Supplementary data can be found at:

https://www.nature.com/articles/s41438-019-0132-8#Sec18

Abstract

Pepper (*Capsicum* spp.) is one of the most important vegetable crops; however, pepper genomic studies lag behind those of other important *Solanaceae*. Here we present the results of a high-throughput genotyping-by-sequencing (GBS) study of a collection of 190 *Capsicum* spp. accessions, including 183 of five cultivated species (*C. annuum*, *C. chinense*, *C. frutescens*, *C. baccatum*, and *C. pubescens*) and seven of the wild form *C. annuum* var. *glabriusculum*. Sequencing generated 6,766,231 high quality read tags, of which 40.7% were successfully aligned to the reference genome. SNP calling yielded 4,083 highly informative segregating SNPs. Genetic diversity and relationships of a subset of 148 accessions, of which a complete passport information was available, was studied using principal components analysis (PCA), discriminant analysis of principal components (DAPC) and phylogeny approaches. *Capsicum annuum*, *C. baccatum* and *C. chinense* were successfully separated by all methods. Our population was divided into seven clusters by DAPC, where *C. frutescens* accessions were clustered together with *C. chinense*. *Capsicum annuum var. glabriusculum* accessions were spread into two distinct genetic pools, while European accessions were admixed and closely related. Separation of accessions was mainly associated to differences in fruit characteristics and origin. Phylogeny studies showed a close relation between Spanish and Mexican accessions, supporting the hypothesis that the first arose from a main genetic flow from the latter. Tajima's D statistic values were consistent with positive selection in the *C. annuum* clusters, possibly related to domestication or selection towards traits of interest. This work provides comprehensive and relevant information on the origin and relationships of Spanish landraces and for future association mapping studies in pepper.

Introduction

Peppers, chilies and ajís, among other terms, refer to different forms of *Capsicum* spp., one of the most important cultivated vegetables in the world (FAO, 2019). Thirty-one species are recognized in the genus, of which twenty-six are wild and five are cultivated (Moscone et al., 2007). The latter are: i) *C. annuum* L., which is the most diverse, economically relevant and studied species, and includes 'bell', 'jalapenos', 'numex' and 'ancho' types, among others; ii) *C. chinense* Jacq., which includes very pungent peppers like the 'habanero' type; iii) *C. frutescens* L. which most known form is 'tabasco'; iv) *C. baccatum* L. or 'ají', which contains the 'lemon drop' and 'ají escabeche' as some of its most common forms; and finally, v) *C. pubescens* Ruiz et Pav., which contains 'rocoto' and 'manzano' types (DeWitt and Bosland, 1996; Kraft, 2009; Nuez et al., 2003). At present, three complexes containing the cultivated peppers are distinguished based on the ability to cross-pollinate: i) *C. annuum* complex, which comprises *C. annuum, C. chinense*, *C. frutescens*, their wild relatives, and *C. galapagoensis* Hunziker (Onus and Pickersgill, 2004), ii) *C. baccatum* complex, which contains *C. baccatum, C. praetermissum* Heiser et Smith and *C. tovarii* Eshbaugh, Smith et Nickrent (Ince et al., 2010; Tong and Bosland, 1999) and iii) *C. pubescens* complex with *C. pubescens* and its wild relatives *C. cardenasii* Heiser et Smith and *C. eximium* Hunziker. Despite the fact that there are strong incompatibility barriers for hybridization among these complexes, the development of viable hybrids, including hybrids between *C. annuum* and *C. baccatum*, has been reported in several works (Manzur et al., 2015; Yoon et al., 2006; Zijlstra et al., 1991).

The cultivated *Capsicum* species encompass a broad diversity as a result of evolution, domestication and artificial and natural selection in agricultural environments in different primary and secondary centres of diversity (DeWitt and Bosland, 1996; González-Pérez et al., 2014). In this regard, Spain is considered a secondary centre of diversity for peppers, especially for *C. annuum* which was brought mainly from Mexico just after the discovery of America (Crosby, 2008). Introduced as an alternative to Asian black pepper (*Piper nigrum* L.), peppers rapidly spread across Europe, Africa and Asia (Nuez et al., 2003). In Spain, a process of more than five-hundred years of selection performed by generations of farmers created a plethora of ecotypes adapted to local conditions, many of which are still cultivated. This is more evident for the fleshy, big-fruited, bell-peppers called 'Morrón', named for their similarity to the nose of a sheep or a cow (i.e. *morro* in Spanish). Amazingly, *C. annuum* fruits and their derivatives have the largest number of EU Protected Designations of Origin (PDO) and Protected Geographical Indications (PGI) in Spain, such as 'Arnoia', 'Pimiento Asado del Bierzo', 'Couto', 'Gernika', 'Morrón de Fresno', 'Riojano' (PGI), and 'Bola – Pimentón de Murcia', 'Padrón', 'Jaranda', 'Pimentón de la Vera', or 'Piquillo de Lodosa' as registered PDO (Nuez et al., 2003; Rodríguez-Burruezo et al., 2016). Despite that, most peppers production in Spain is based on F1 hybrids from 'California Wonder',

'Lamuyo' and 'Dulce Italiano' types, which have displaced the traditional and ancient materials mentioned earlier. However, the interest for the "taste of the past" by consumers and the challenge of adapting to climate change are contributing to the enhancement and reintroduction of landraces (Brugarolas et al., 2009; Hammer et al., 2003; Rodríguez-Burruezo et al., 2016). In addition, the studies on diversity of these materials are of importance in terms of: i) genetic fingerprinting of varietal types, ii) registration of materials, and farmers and communities rights preservation, iii) genetic relationships in order to provide breeders with information about the available materials for breeding programs, iv) conservation of genetic resources, v) development of non-redundant core collections, and vi) revert the variability loss due to genetic erosion.

Despite its economical relevance, the development of *Capsicum* molecular tools lags behind other economically important *Solanaceae* crops, such as tomato or potato (Ashrafi et al., 2012; Qin et al., 2014). Its unusually large genome and repetitiveness may be the reasons for this delayed development (Kim et al., 2014; Park et al., 2012; Qin et al., 2014). In this respect, germplasm diversity analysis is one of the key elements for plant breeding and biodiversity conservation of *Capsicum* species (Prohens et al., 2017; Yoon et al., 2006). However, it is highly dependent on the availability of informative genetic tools such as molecular markers (Ibiza et al., 2012; Ince et al., 2010).

Throughout the last decade, high-throughput sequencing technologies development was stimulated by the need for low cost data and by the availability of faster analysis tools (Glaubitz et al., 2014; He et al., 2014). High-throughput Genotyping-by-Sequencing (GBS) has had an important impact on the scientific community due to its versatile application (Chung et al., 2017; Gardner et al., 2014; He et al., 2014; Poland and Rife, 2012). This approach can provide accurate results independently of the target species or population, and does not require having previously available genomic information (Elshire et al., 2011; He et al., 2014; Poland and Rife, 2012). GBS has been successfully used in pepper in recent years and an important amount of highly informative genome-wide SNPs were generated in each experiment. Germplasm diversity, population structure and genomic selective sweeps analysis, as a result of domestication or local adaptation, are common to these works as an important first step for latter Genome-Wide Association Studies (GWAS) (Taitano et al., 2018; Taranto et al., 2016). GBS-generated SNPs have been proven useful in the detection of trait-associated QTLs for both *C. annuum* and *C. baccatum* paving the way for further association studies and for a better understanding of pepper's evolution (Nimmakayala et al., 2016b, 2016a). To our knowledge, our work is the first to use GBS to analyse population structure and diversity and to assess selective genomic sweeps in a collection of Spanish landraces.

Herein we present a diversity study of a collection of *Capsicum* spp., encompassing four cultivated species and the *C. annuum* wild ancestor *C. annuum var. glabriusculum* using GBS. Our goals are genotyping a representative collection of Spanish heirlooms and ecotypes encompassing most varietal types of pepper, to shed light into the Spanish

landraces' phylogenetic relationships, among them and with other materials, to evaluate their molecular diversity and population structure.

Material and methods

Plant material

A diverse collection of 190 *Capsicum* spp. accessions encompassing five cultivated species, *C. annuum* var. *annuum* (from now on *C. annuum*; 137 accessions), *C. chinense* (14 accessions), *C. frutescens* (2 accessions), *C. baccatum* (28 accessions), *C. pubescens* (2 accessions), and the wild form *C. annuum* var. *glabriusculum* (7 accessions), commonly known as 'chiltepín', was considered for GBS sequencing.
We report here the sequencing results for the collection mentioned above, although, since for 42 of the 190 accessions we didn't possess a complete passport information those were excluded for downstream analysis. Hence, the 148 accessions subset was provided by the Universitat Politècnica de València Germplasm Bank, the COMAV *Capsicum* breeding group (112 accessions), several other research institutions (e.g. Institut National de la Recherche Agronomique (INRA-GEVES), Maritsa Vegetable Crops Research Institute (MVCRI), Mexico Chile breeding program of Universidad Autónoma de Aguascalientes (UAA), Penn State University and United States Department of Agriculture (USDA); 23 accessions) and several seed companies (e.g. Batlle, Franchi Simenti, Intersemillas, Mascarell, Ramiro Arnedo, Reimer Seeds, and Zeraim Ibérica; 13 hybrids and heirloom lines) (Supplementary Data: Table 1).

The considered subset encompassed *C. annuum* (118) accessions, *C. annuum* var. *glabriusculum* (7), *C. chinense* (12), *C. frutescens* (2) and *C. baccatum* (9) (Supplementary Data: Table 1). Most of the considered accessions from *C. annuum* correspond to sweet, red, bell-shaped Spanish landraces, although a considerable amount of variability for pungency/sweetness, colour, fruit shape, resistances, origin, and varietal types was also included for a better evaluation of the diversity and phylogeny of the *Capsicum* genus (Supplementary Data: Table 1). Spanish *C. annuum* heirlooms and traditional materials have been prospected for more than 35 years in all regions of the country and, therefore, they can be considered highly representative of the variation of this centre of diversity in this species.

DNA extraction, library preparation and sequencing

DNA was extracted from young leaves using a modified CTAB protocol (Doyle and Doyle, 1990). Raw and restriction enzyme *Hind*III (Thermo Fisher Scientific, Wilmington, North Carolina, USA) digested DNA electrophoresis was run on 0.8% agarose gel to assure DNA integrity. Purity was assessed using Nanodrop® (ND-1000, Thermo Fisher Scientific, Wilmington, North Carolina, USA) and quantity was assessed with Qubit™ (2.0 Fluorometer, Invitrogen, Carlsbad, California, USA). High molecular weight DNA aliquots with 230/260 and 260/280 ratios ranging between 1.8-2.0 and 1.8-2.2, respectively, were then sent to Cornell University sequencing facilities (Ithaka, New York, USA). *Ape*KI methylation-sensitive restriction enzyme was used for library preparation as described by Elshire et al. (2011). Illumina sequencing adapters and sample-specific barcodes were then ligated to the resulting fragments sticky ends and samples were pooled together for multiplexing. PCR (Polymerase Chain Reaction) was performed for library construction and Illumina HiSeq2500 (Illumina, Inc., San Diego, California, USA) single-end technology was used for library sequencing. Generated good barcoded reads were captured, collapsed by similarity and stored into a FASTQ file to generate unique tags. Only tags occurring at minimal count (>=3) were retained to generate a MasterTag file (FASTQ) as described by Elshire et al. (2011) and Glaubitz et al. (2014).

Mapping and SNP calling

MasterTag file was aligned against the reference genome CM334 (Criollo de Morellos version 1.55) (Kim et al., 2014) using Burrows-Wheeler Aligner (BWA version 0.7.8-r455) (Li and Durbin, 2009) set to default settings. Aligned sequence tags were stored into TOPM (TagsOnPhysicallMap; SAM/BAM format (Li et al., 2009)) file. SNP calling was then performed applying TASSEL-GBS Pipeline (version 3.0.173) (Bradbury et al., 2007; Glaubitz et al., 2014). Low quality SNPs were filtered out by minimum minor allele frequency (mnMAF<0.01) and missing data per site (MDpS>10%), and finally converted into Variant Call Format file (VCF).

Sequencing and SNP calling statistics

Sequencing, alignment, SNP calling and population statistics were performed for a better understanding of results quality. SAMtools (version 1.8) (Li et al., 2009) was used to calculate the number of reads that passed the quality control and aligned successfully against reference. BEDtools (version 2.25.0) (Quinlan and Hall, 2010) was used to assess the percentage of sequence tags that overlap with genic regions. Transitions/transversions ratio was obtained by BCFtools (version 1.8) (Li et al., 2009).

And finally, heterozygosity was obtained by VCFtools (version 0.1.14) (Danecek et al., 2011).

Population structure analysis

Evolutionary relationships and population structure were analysed implementing Rstudio (version 1.1.383) (R Development Core Team, 2009). As a first step, VCF file resulting from SNP calling was subjected to another filtering process by SNPRelate package (version 1.12.2) (Zheng et al., 2012). Steps included: 1) removing multi-allelic, monomorphic, and low-quality positions; and, 2) filter SNPs with a linkage disequilibrium (LD) threshold of 0.2. Initial analysis of population structure was carried out by principal components analysis (PCA) using the SNPRelate package (Zheng et al., 2012) and plotted using ggplot2 package (version 2.2.1) (Wickham, 2016).

To better understand and describe the genetic structure, discriminant analysis of principal components (DAPC) was applied using DAPC function from adegenet package (version 2.1.1) (Jombart, 2008; Jombart et al., 2010; Jombart and Ahmed, 2011). Most informative SNPs obtained by SNPRelate (Zheng et al., 2012) were used as input. DAPC workflow consisted of: 1) dataset transformation based on PCA, 2) determination of the optimal number of clusters by Bayesian information criterion (BIC) for K=1 to 20 by k-means clustering with 100,000 iterations and 1,000 randomly chosen starting centroids, 3) selection of K with lowest BIC as optimal number of clusters, selection of optimal number of PCs and discriminant analysis (DA) functions to retain, and lastly 4) DAPC computation and plotting using ggplot2 (Wickham, 2016).

For further elucidation of genetic distance between samples and clusters, a phylogenetic tree was constructed. For that, the aboot function from Poppr package (version 2.7.1) (Kamvar et al., 2014) was run with the parameters bitewise.distance tree with neighbour-joining algorithm (Saitou and Nei, 1987) and 1,000 bootstrap replicates. Plot.phylo function loaded from ape package (version 5.0) (Paradis et al., 2004) plotted the generated tree.

Genetic diversity and selective sweeps

Population genetic diversity was calculated through Weir and Cockerham's F_{st} index (Weir and Clark Cockerham, 1984) between all the clusters detected by DAPC. Tajima's D statistic (Tajima, 1989) with a bin size of 500kb was used to identify selective sweeps from our data for all the established clusters and plotted with ggplot2 package (Wickham, 2016). The mentioned statistics were obtained by VCFTools (Danecek et al., 2011).

Results and discussion

Sequencing and SNP calling

A collection of 190 *Capsicum* spp. accessions was successfully sequenced using the Illumina HiSeq2500 platform and yielding a total of 568,964,449 raw reads of which 524,450,716 (92.18%) represented good barcode reads. FASTQ file containing only collapsed and filtered reads was then aligned against the CM334 reference genome (Kim et al., 2014). From the 6,766,231 unique read tags present in the file, 40.8% uniquely aligned, 7.4% multiply aligned, and 51.8% did not align successfully to the reference genome. Only 40.8% of sequences uniquely aligned to the reference genome, considerably fewer than the ones that do not align. These results could be due to 1) lack of reference for some positions in the reference genome, 2) the repetitiveness inherent to pepper genome contributing to two or more aligning sites for some sequences, 3) several accessions from species other than *C. annuum* probably could not align correctly, 4) some of the produced sequences could be mitochondrial or chloroplast DNA. The results are consistent with other recent works with this genus. Taranto et al. (2016) aligned uniquely 43.4% of tags and 9.8% aligned to multiple sites; and Ahn et al. (2018) obtained 45.5% of uniquely aligned sequence tags for a *C. baccatum* accession and 39.2% for a *C. annuum* accession (Ahn et al., 2018).

*Ape*KI was selected for genome complexity reduction due to its sensitivity to methylation and consequent frequent cut in gene-rich regions (Elshire et al., 2011; Sonah et al., 2013). BAM file containing only mapped reads was compared against the previously available CM334 annotated genes gff3 (General Feature File) file in order to calculate the number of intersected genic regions. From a total of 3,260,848 tags, 39.3% overlapped genic regions. Our results indicate that chromosomes 2 (49%), 3 (48%) and 8 (47%) have the greater percentage of tags on genic regions (Figure 1). Contrastingly, chromosomes 9 (33%), 10 (32%) and 11 (32%) presented the lowest values of tags overlapping genes, with only a third of total number of read tags being located inside these regions (Figure 1). Taranto et al. (2016) using GBS technology and a widely diverse collection reported similar results. These results could be due to the GBS protocol, which targets preferably genic regions and has a lower genomic coverage especially for repetitive regions (Hulse-Kemp et al., 2018; Sonah et al., 2013).

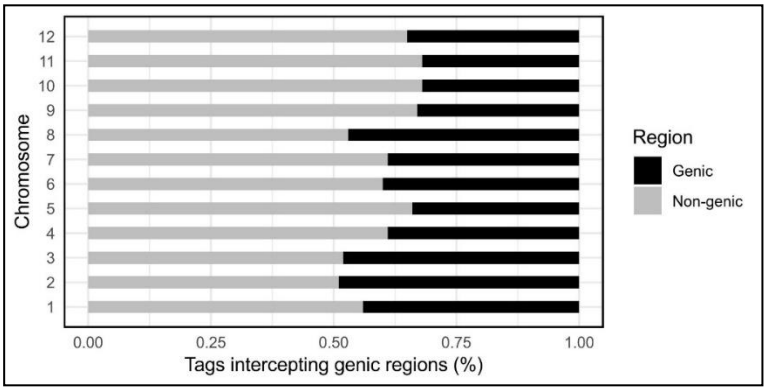

Figure 1 – Distribution of filtered Illumina sequencing tags intercepting genic (black) and non-genic (grey) regions per chromosome.

Variant calling was performed by TASSEL-GBS Pipeline (Bradbury et al., 2007; Glaubitz et al., 2014) on the TOPM (BAM) file containing only uniquely aligned sequences producing 640,377 raw SNPs. Finally, after removing low quality positions by MAF and MDpS, 531,680 SNPs, distributed along the entire genome, were selected (Supplementary Data: Figure 1).

Transitions were found in greater proportion (59.7%). Transitions/transversions ratios are in agreement to other previous reports for pepper (Taranto et al., 2016). This phenomenon could be explained as an evolutionary advantage in case of mis-pairing because they are more likely to preserve protein structure than transvertions (Wakeley, 1996).

The levels of observed heterozygosity for the 531,680 called SNPs ranged between 2.35 to 6.50% for these pepper accessions and averaged 3.16%. Regarding species, *C. annuum var. glabriusculum* (2.87%) displayed the lowest mean value, while *C. baccatum* (5.20%) the highest. And finally, experimental lines (3.10%) presented a lower mean value of observed heterozygote positions, while commercial hybrids (4.15%) presented the highest (Supplementary Data: Table 2). Values as low as the ones found here are not unusual for autogamous species such as pepper (Eshbaugh, 1975; Raw, 2000). Lower mean values for observed heterozygosity were reported by Taranto et al. (2016) for a collection of 397 accessions from 8 different species (2.40%). However, Cheng et al. (2016) and Lee et al. (2016) reported higher mean values for bigger and more diverse populations than ours, 17.00% (ranging from 1.00 to 23.00%) and 15.00% (ranging from 9.00 to 21.00%), respectively. In another study, Nimmakayala et al. (2016a) reported values in between the ones mentioned above (6.00%, ranging from 3.00 to 18.00%) for a diverse *C. annuum* population. The

literature seems to support the perception that wild accessions have higher heterozygosity values than cultivated (Lee et al., 2016). In this way, Ibiza et al. (2012) found a higher level of heterozygosity for *C. baccatum*, possibly indicating a higher level of allogamy than the others (Supplementary Data: Table 2).

SNP filtering

A previous step to remove low quality and monomorphic positions by mnMAF, MDpS and LD was performed. Original VCF with 531,680 positions was filtered by SNPRelate package (Zheng et al., 2012) resulting in a significant decrease to 4,083 highly informative and well distributed across genome variants (Supplementary Data – Figure 1; Supplementary Data – Table 3). Most pepper population diversity and structure analyses have relied on just a dozen to a few dozens of markers due to lack of data resolution of the pepper's genome (González-Pérez et al., 2014; Lee et al., 2016; Nicolaï et al., 2013). Fortunately, with the NGS technologies, thousands of markers are now easily available for researchers (Elshire et al., 2011). Thus, the most recent works used a similar number of markers to our study in order to assess genetic structure (Taitano et al., 2018) and for GWAS (Nimmakayala et al., 2016a).

Population genetic relationships

The set of 4,083 SNPs and SNPRelate package (Paradis et al., 2004) were used for PCA analysis. The first two principal components (PC) accounted for 45.5 and 6.3% of total variability, respectively (Figure 2). The population can be divided into three different clusters: i) one comprising all *C. annuum* plus four *C. annuum var. glabriusculum* accessions (mex_v1196, mex_q1078, mex_s1120 and usa_a1003), ii) a second composed exclusively of *C. baccatum* accessions and iii) a plurispecific cluster formed by *C. chinense*, *C. frutescens* and the remaining three *C. annuum var. glabriusculum* accessions. The first PC (PC1) separated species by complexes. In this way, the *C. annuum* complex (*C. annuum*, *C. chinense* and *C. frutescens*) accessions displayed low and negative values for the PC1 whereas the *C. baccatum* complex accessions showed positive values. The second PC (PC2) differentiated *C. annuum* from the other species. Several studies using SSR (González-Pérez et al., 2014; Nicolaï et al., 2013) and SNP markers (Lee et al., 2016; Taranto et al., 2016) described a similar separation of species. As in our case, Nicolai et al. (2013 and González-Pérez et al. (2014) reported a close relationship between *C. chinense* and *C. frutescens*. *Capsicum annuum var. glabriusculum* peculiar distribution was also mentioned in Nicolai et al. (2013 and Taranto et al. (2016), where it was positioned near *C. annuum*, *C. chinense* and *C. frutescens*.

In order to visualize in detail, the relationships within the *C. annuum* cluster, a PCA was performed with only those accessions and the four closest *C. annuum var. glabriusculum* accessions, comprising 122 accessions. The PC1 (14.5%) and PC2 (8.6%) separated the accessions based on origin and fruit traits (Figure 2). Sweet large-fruited Spanish and other European accessions (Bulgaria, France, Italy and Serbia) clustered together, whereas pungent small-fruited North American (Mexico and USA) and Indian, Spanish, Turkish, and Sri Lankan accessions represented a much more diverse cluster of accessions. *C. annuum var. glabriusculum* mex_s1120 and usa_a1003 accessions clustered together and far away from the *C. annuum* group, mex_q1078 was at mid-distance from groups, and mex_v1196 was the closest to the cluster formed by the pungent *C. annuum* varieties (Figure 2). Nicolai et al. (2013), Lee et al. (2016) and Taranto et al. (2016) reported a similar distribution based on fruit traits or geographic origin inside the *C. annuum* cluster.

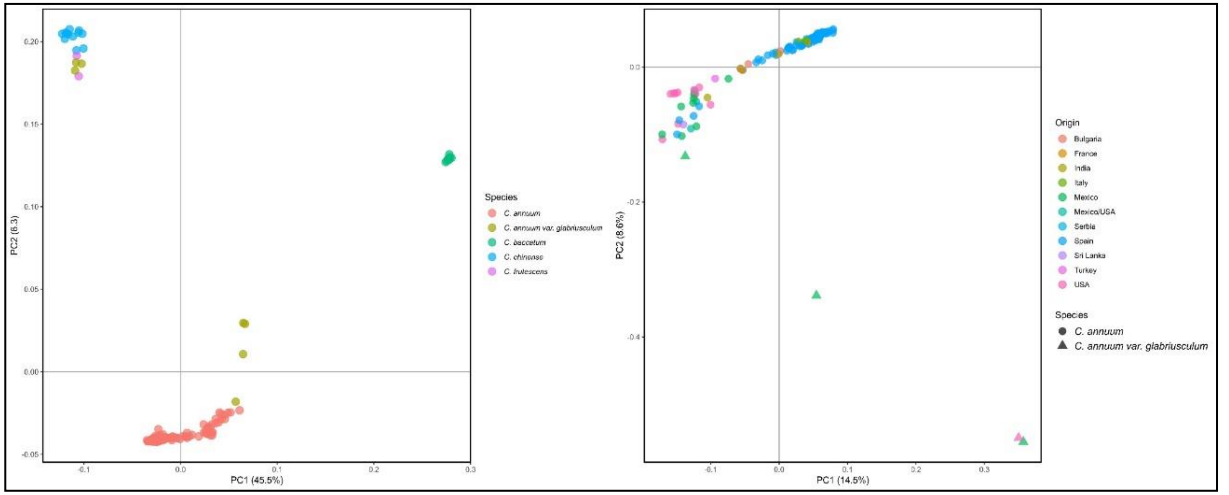

Figure 2 – First and second principal components for both 148 accessions (left) and 118 *C. annuum* and closely related four *C. annuum* var. *glabriusculum* (right) based on 4,083 filtered SNPs. Accession corresponding species (left) and origin (right) information is provided in a colour palette in each graph.

Population genetic structure

For further elucidation of the genetic structure DAPC was pursued. K values and lineal components to be retained were pre-determined using the find.clusters function. K=7 was determined to be the most likely as indicated by BIC value (Supplementary Data – Table 4). The first 100 PCs and the first two DA functions were retained for the analysis, representing more than 90% of total variability. Notwithstanding, both K=6 and K=8 showed similar BIC values and cluster formation meaning that could also represent a good fit to our collection (Supplementary Data – Table 4).

DAPC results were similar to those obtained by PCA, although with much more detail. Our population seems to be separated into seven clusters (Figure 3). Cluster 1 comprised a set of 39 accessions, most of them Spanish landraces and 3 other European accessions (France, Italy and Serbia) classified as *C. annuum* and sharing similar fruits traits: sweet, red, blocky peppers with variable fruit size and flesh thickness. Cluster 4 includes a diverse group of accessions composed of 44 European accessions, mostly *C. annuum* Spanish landraces and others with European origins (Bulgaria, France and Italy) representing sweet, big sized, blocky peppers. Both Clusters 1 and 4 showed admixture and a narrow range of diversity between them. This was also observed by Nicolai et al. (2013), Lee et al. (2016) and Taranto et al. (2016) and the explanation probably resides on the fact that most Spanish varieties probably descend from a restricted number of individuals brought since Columbus journeys to the Americas and then spread across several countries which through selection and adaptation to local conditions originated a new range of forms (Andrews, 1995; DeWitt and Bosland, 1996; Nuez et al., 2003). Another reason why it is so difficult to differentiate European accessions may be the introduction of the same commercial lines in many different areas and this might be changing the genetic structure by cross pollinate with local varieties (Ibiza et al., 2012). Cluster 2 was formed by 22 accessions, all of them *C. annuum* and encompassing a great diversity of places of origin. Most accessions in this cluster were collected in North American territories (Mexico and USA) with several fruit shapes. Europe was also represented with seven accessions from Bulgaria, France and Spain, and finally one accession from India and another from Turkey. Bulgarian and French accessions were the only ones non-pungent; however, its fruit shape suggested that they could be improved lines developed from Mexican materials. Besides geographic origin, pungency could be a defining trait for population structure (González-Pérez et al., 2014; Taranto et al., 2016). A group of 14 pungent accessions formed Cluster 3, including 13 *C. annuum* and one *C. annuum var. glabriusculum*. Most of them are Mexican varieties with cayenne and jalapeno shaped fruits and is completed by three Spanish accessions, one from Sri Lanka and one from USA, all pungent and with the same fruit shape. *C. annuum var. glabriusculum* is thought to be an ancestor to the Mexican *C. annuum* (Hayano-Kanashiro et al., 2016; Moscone et al., 2007) so its presence in this cluster is plausible and could be affected by the gene flow between domesticated forms and this

botanical variety. Another hypothesis is that it could be a misclassification since *C. annuum* has an important range of phenotypes that could lead to an error during classification.

As in the PCA, all nine accessions identified as *C. baccatum* clustered together into a clearly differentiated group (Cluster 5; Figure 3). This set of accessions seems to have a particular genetic print that makes them different from the rest of considered species. *Capsicum baccatum* was domesticated independently from the other species in South America and it is still cultivated in isolated mountain areas that difficult the gene flow with other populations (Ibiza et al., 2012; McLeod et al., 1982; Pickersgill, 2007). In addition, its crossability with species outside of its cytogenetic complex is difficult (Walsh and Hoot, 2001). Ibiza et al. (2012) reported a separation between Bolivian and Peru/Ecuador *C. baccatum* accessions corroborating that geographic isolation is an important factor for genetic structure.

Cluster 6 was composed of only three *C. annuum var. glabriusculum* accessions (mex_q1078, mex_s1120 and usa_a1003) that seemed to have a similar genetic print and were separated from the rest of accessions classified as the same species (Figure 3). Nicolai et al. (2013) and Taranto et al. (2016) were not able to allocate this species into any group. The former reports three distinct genetic pools for this botanical group.

Finally, *C. chinense* and *C. frutescens*, as well as three *C. annuum var. glabriusculum* accessions (mex_c1333, mex_n1411 and mex_o1430), appeared to be indistinguishable accessions and were assigned to Cluster 7 as also seen in the PCA plot. The group includes 17 accessions from several regions of South and North America. Fruits are typically pungent, small sized and with several fruit shapes. Many authors agree that *C. chinense* and *C. frutescens* should be considered as a single species (McLeod et al., 1979; Walsh and Hoot, 2001) and our results may support this hypothesis. However, *C. frutescens* was represented by only two accessions so solid conclusions could not be drawn. It is believed that a common ancestor through domestication in different locations gave place to *C. annuum*, *C. chinense* and *C. frutescens* and the close relation seen in this work and others indicates that *C. annuum var. glabriusculum* could be that link (Moscone et al., 2007; Nicolaï et al., 2013).

DAPC was also performed for *C. annuum* and the four *C. annuum var. glabriusculum* closest accessions. K=5 was determined as the optimal number of clusters and the first 100 PCs and the first two DA functions were retained for the analysis (Figure 3). Other Ks presented a possible good fit as both K=4 and K=6 showed slightly higher BIC values (Supplementary Data – Table 4). Results seem to be in agreement with the ones observed in the DAPC with all the collection and the formed clusters were homologous between analyses (Figure 3). DAPC clusters 1 to 5 for the 122 accessions corresponded to the DAPC clusters 6, 2, 3, 4, 1 for 148 accessions, respectively, the only difference being that admixture samples from the first plot are now two clearly distinct groups. The

clusters 1 to 5 of *C. annuum* are now composed of 3, 22, 14, 44 and 39 accessions, respectively (Supplementary Data – Table 4).

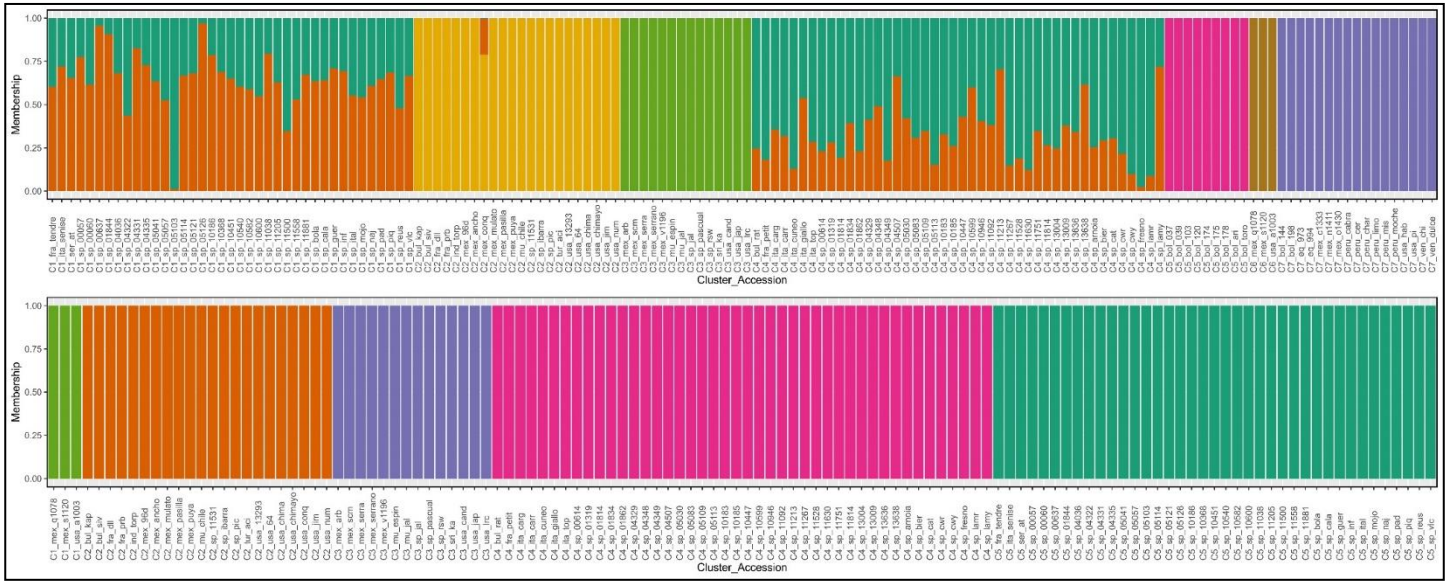

Figure 3 – Population structure for both 148 considered accessions (top) and subset of 122 *C. annuum* accessions (bottom) given by DAPC with 4,083 informative sites. Colours represent different assigned clusters. The *x*-axis provides accession names and respective assigned cluster whereas the *y*-axis provides the probability of each accession belonging to the assigned cluster.

Population phylogenetic relationship

A phylogenetic tree for all the 148 accessions and another one only for the 122 *C. annuum* accessions were constructed for a better visualization of sample distribution and relationships (Figures 4 and 5).

Capsicum annuum clustered separately from the rest of species, forming a large cluster. This is in agreement with previous results (Ibiza et al., 2012; Lee et al., 2016; Nicolaï et al., 2013). Genetic distance increased from European to North American accessions. As said before, Spanish varieties arose from the ones brought from Mexico since Columbus journeys and were bred into a range of different forms (Andrews, 1995; DeWitt and Bosland, 1996; Nuez et al., 2003). This explains the genetic closeness of many Spanish accessions to Mexican materials. From Spain it spread across Europe and therefore the difficulty to cut apart European lines (Nicolaï et al., 2013; Taranto et al., 2016).

C. annuum var. glabriusculum accessions appeared scattered in the dendrogram. Four out of seven accessions appeared along the *annuum-chinense-frutescens* complex, of which mex_v1196 and mex_q1078 accessions were especially close, whereas mex_s1120 and usa_a1003 located further to the *C. annuum* clade. The remaining three accessions (mex_c1333, mex_n1411 and mex_o1430) located more closely to *C. chinense* and *C. frutescens* accessions (Figure 4), hence indicating a possible link between those species and a possible common ancestry (Moscone et al., 2007; Nicolaï et al., 2013).

Capsicum chinense and *C. frutescens* grouped together into a clearly separated cluster (Figure 4). In our case, the sample size was not large enough to make strong assumptions, given that *C. frutescens* was represented by only two accessions, but our findings seem to reinforce that *C. chinense* and *C. frutescens* should be considered the same species (McLeod et al., 1979; Walsh and Hoot, 2001).

Finally, the *C. baccatum* cluster located between the *C. annuum* main cluster and the *C. chinense-C. frutescens* group, although considerably closer to the second (Figure 4). At first sight, these findings disagree with the works from González-Pérez et al. (2014) and Nicolai et al. (2013), who reported *C. frutescens* as the closest species of *C. annuum* followed by *C. chinense* and finally *C. baccatum*, or alternatively, Lee et al. (2016), who reported a slightly closer relation between *C. annuum* and *C. chinense*. Both groups of species, i.e. *C. baccatum* and *C. chinense-C. frutescens*, were domesticated independently but in relatively close regions, i.e. *C. baccatum* ancestors migrated to the south of Bolivia while the ancestral genetic flow of *C. chinense* and C. *frutescens* migrated towards the Amazonian basin where these species were domesticated (Moscone et al., 2007; Pickersgill, 2007). By contrast, *C. annuum* arose farther away, in Mexico (Kraft et al., 2014). Thus, this geographical compartmentalization might explain

why our *C. chinense* and *C. frutescens* are closer to *C. baccatum* than *C. annuum* (Moscone et al., 2007; Pickersgill, 2007).

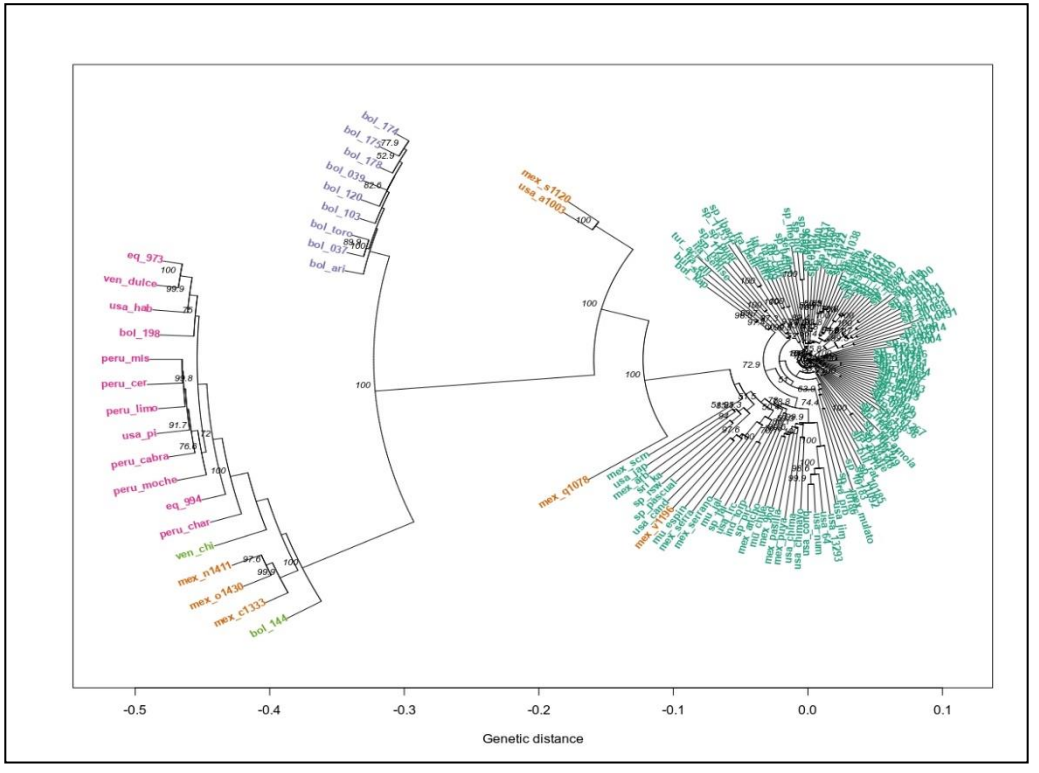

Figure 4 - Clustering tree created from 1000 bootstrap replicates for the 148 accessions considering 4,083 filtered SNPs.
Dark green indicates *C. annuum* accessions, whereas *C. annuum var. glabriusculum*, *C. baccatum*, *C. chinense* and *C. frutescens* accessions are represented in orange, purple, pink and light green, respectively. Node values correspond to bootstrap values.

Considering the tree containing only *C. annuum*, two groups can be identified: one mainly grouping Mexican and USA accessions, including the four chiltepíns (mex_s1120, usa_a1003, mex_q1078, and mex_v1196) and another including mostly *C. annuum* materials from Spain (Figure 5).

In the first cluster, we found that 'Chile Japones' (usa_jap), several 'serrano' forms (mex_scm, mex_serra and mex_serrano), 'jalapenos' (usa_cand, mu_espin, mu_jal and sp_jal) and 'Chile de Arbol' (mex_arb) grouped the most closely to (wild) chiltepíns, as well as some pungent, thin-flesh cayennes like the Spanish 'Guindilla Pascual' (sp_pascual), 'Picante Largo' (sp_pic), the breeding line 'RSW' and the Indian 'Torpedo of Bangalore' (ind_torp) (Figure 5). Our results seem to agree that 'serrano' peppers are close to the ancestral forms within the cultivated *C. annuum*. In fact, they share some wild traits such as pubescence and soft flesh deciduous fruits (Bosland and Votava, 2012) and 'Jalapeno' peppers were mainly bred from serrano gene pools, thus the closeness.

In the second cluster, the closest materials to the North American accessions were the Turkish pungent 'Aci Sivri' (tur_aci) and sweet numex-like Bulgarian accessions (bul_siv and bul_kap), followed by another small cluster which grouped Basque 'Guindillas' (cayennes; sp_11531 and 'sp_ibarra'), 'Guernika' (sp_guer), 'Padron' (sp_11205 and sp_pad) and 'Peperone di Senise' (ita_senise). These results show a close relationship among Turkish and Balkan materials, suggesting common genetic pool or historic exchange of materials between both countries. The phylogenetic proximity of some cayenne peppers like 'Aci Sivri' or 'Guindilla de Ibarra' to North American peppers like 'Chile de Arbol' was supported by their similarity in the pattern of volatiles (Rodríguez-Burruezo et al., 2010). In addition, these findings are in agreement with the history of Padron peppers, which were brought to Galicia (Spain) by the Franciscans from Mexico in the XVIIth century. This flow of materials from Mexico might have included also the ancestors of many ecotypes from other Northern Spanish regions like 'Guernika', with a similar fruit appearance to Padron peppers (Rodríguez-Burruezo et al., 2016).

Triangular-shaped Spanish materials from northern Spain (sp_10183, sp_10185, sp_10186 and sp_11092) and the Mexican 'Mulato' (mex_mulato) clustered closely to North American accessions, suggesting that Ancho/Poblano peppers from Mexico might be the ancestors of Piquillo peppers as suggested by Rodriguez-Burruezo et al. (2010) (Figure 5).

The rest of *C. annuum* materials, mainly bell peppers, grouped in several subclusters. 'Cuneo' (ita_cuneo and ita_giallo) and 'Carmagnola' peppers (ita_carr and ita_carg), from the Italian Piedmont and characterized by large and slightly flattened 'Morron' (blocky) peppers, grouped with the French 'Petit Marsellais' (fra_petit) (Figure 5). At first sight this is very surprising because 'Petit Marsellais' is a yellow-orange thin-fleshed small-fruited heirloom from the French Provence. However, its fruits look like

small blocky peppers and, therefore, a few mutations relative to the fruit size and flesh thickness (Wang et al., 2015) and/or the geographical proximity between the Provence and Piedmont might have enabled some genetic exchange.

Close to the subcluster of Italian-Piedmont peppers we also found other two groups: a small subcluster which includes 'Pimiento de Mojo' (sp_mojo), 'Piquillo' PGI (sp_piq) and 'Bola' PDO (sp_bola) and another larger group of Spanish 'Morron' peppers which encompasses materials from accession sp_05057 to accession sp_10582 (Figure 5). Accessions in the first subcluster shared thin flesh and high dry matter content, useful for their culinary uses: the mojo picon (hot sauce) in Canary Islands, roasted and canned to be stuffed and ground to obtain pepper powder, respectively (Nuez et al., 2003; Rodríguez-Burruezo et al., 2016). The second subcluster can be divided further into two groups of 'Morron' peppers. One group (from accessions sp_5057 to sp_11038) of large 'Morron' peppers (most fruits ≥150 g) from several Spanish regions; and a second group which comprises most 'Valenciano' accessions from Valencia and Murcia regions (sp_4331, sp_5030, sp_05103, sp_5121, sp_5126, sp_vlc and sp_10582) as well as 'Largo de Reus' peppers from Catalonia (sp_reus, sp_01844 and sp_10600) (Figure 5). Thus, despite a few accessions using these names can be found in other clusters (e.g. accessions sp_01862, sp_05113), these findings suggest a common genetic pool linked to such varieties along the Mediterranean coast of Spain (from Murcia to Catalonia), which can offer the opportunity of finding genetic fingerprints associated to the 'Valenciano' or 'Largo de Reus' denominations in the next future.

Finally, other four interesting subclusters are worth mentioning: i) one ranging from accessions sp_cala to sp_10451, ii) from accessions sp_11630 to sp_04329, iii) from sp_11751 to sp_01319, iv) from sp_bier (PGI Pimiento Asado del Bierzo) to sp_10599 (Figure 5). The first encompasses most accessions from a particular type of 'Morron' peppers, commonly called 'Morron de Conserva' or 'Morron de Bola', with characteristic round/heart-shaped fruits ('bola' in Spanish) (Supplementary Data: Table 1). These varieties have been selected for being roasted and canned ('conserva' in Spanish) and, despite the lack of nose-shape of the true Morron peppers, they keep the term 'Morron' because of their thick flesh (Rodríguez-Burruezo et al., 2016). Calahorra peppers from La Rioja are considered the ancestors of this kind of Spanish peppers, which can be found throughout the country (Figure 5). The second includes a miscellany of 'Morron' peppers from very different origins like breeding lines from seed companies and our research institute from Valencia, i.e. 'California Wonder' and 'Lamuyo' (modern 'Morron'), and some 'Valenciano', 'Largo de Reus' and 'Trompa de Vaca' peppers (Figure 5). With the only exception of sp_11603, all of them are from the Mediterranean coast of Spain, which suggests that at least two different lineages of 'Morron' peppers, the one with most 'Valenciano' and 'Largo de Reus' peppers and this one, are present in this region. The third and the fourth subclusters include 'Morron' peppers from the North of the country, but the former from Pyrenees to Cantabric Sea regions, while the latter from Leon and Zamora including two current PGI and ancient

related ecotypes: PGI ‘Fresno de la Vega y Benavente’ (sp_fresno) and PGI ‘Pimiento Asado del Bierzo’ (sp_bier) (Supplementary Data: Table 1). Both PGI belong to different Pochard´s types, which suggests that genetic exchanges might have occurred among both varietal types in this region.

The three methods considered were able to detect, although with different levels of detail, the complex relations among the collection, and seemed to be coherent with each other. *Capsicum annuum*, *C. baccatum* and *C. chinense* separation was observed in all, as well as the incorporation of *C. frutescens* accessions into *C. chinense* cluster, and *C. annuum var. glabriusculum* distribution into two distinct genetic pools. Both PCA (Figure 2) and DAPC (Figure 3) offered an idea of the genetic structure behind the collection, however, phylogenetic tree (Figures 4 and 5) gave a greater level of details on the relations among species and even among closely related accessions

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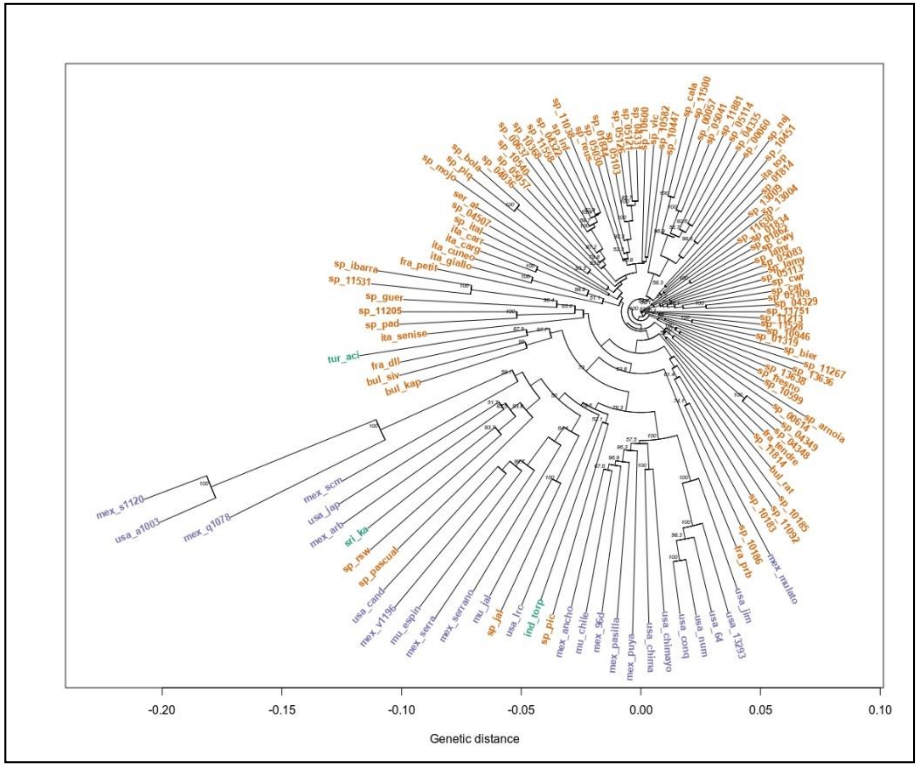

Figure 5 - Clustering tree created from 1000 bootstrap replicates for the 118 *C. annuum* and closest four *C. annuum* var. *glabriusculum* accessions. Green indicates Asian accessions, orange the European, and purple the North American accessions. Node values correspond to bootstrap values.

Genetic diversity among clusters

The average weighed F_{st} value between the seven clusters was 0.486. The highest F_{st} value was observed between clusters 4 and 7 (0.739) and the lowest between 1 and 4 (0.069) (Table 1). Low values indicate a larger genetic difference among accessions intra-population than between populations, suggesting genetic flow between populations. High values suggest low genetic flow between populations and many genetic differences (Holsinger and Weir, 2009; Weir and Clark Cockerham, 1984).

Spanish and other European accessions (Clusters 1 and 4) presented a lower level of genetic differentiation, suggesting a genetic flow between populations (Table 1). Similar results were reported by Nicolai et al. (2013), Lee et al. (2016) and Taranto et al. (2016). This is probably due to the founder effect by a narrow diversity brought from the New World (Andrews, 1995; DeWitt and Bosland, 1996; Nuez et al., 2003).

Cluster 4 and 7 had the highest F_{st} value among all cluster combinations (Table 1). The first is mainly composed of European *C. annuum* accessions with low genetic variability whereas the second is an interspecific cluster composed of *C. chinense*, *C. frutescens* and *C. annuum var. glabriusculum*. Both clusters are geographically separated and the accessions have no genetic flow so the high F_{st} value is consistent with that scenario.

Cluster 5 also presented high F_{st} values regarding all combinations, which reveals that the population is isolated and has none or low genetic flow with other clusters, as expected for its geographic origin (Supplementary Data: Table 1). *Capsicum baccatum* cultivation usually takes place in isolated areas that difficult crosspollinations. Furthermore, crosses outside its botanical complex are extremely difficult (Ibiza et al., 2012; McLeod et al., 1979; Walsh and Hoot, 2001).

Our data is consistent with previous published data. Taitano et al. (2018) reported a mean F_{st} of 0.821, ranging from 0.199 to 0.952, between 5 *C. annuum* landraces and *C. frutescens*. Nimmakayala et al. (2016b) presented a fixation index of 0.780 between cultivated *C. annuum* and *C. baccatum* and 0.660 between wild accessions of those same species. Nimmakayala et al. (2016a) reported for an only exclusively *C. annuum* with distinct levels of pungency and fruit weight an F_{st} between 0.020 and 0.150.

Table 1 - Weighed pairwise F_{st} values for the seven previously determined clusters. Colours indicate an increasing number of genetic differences between clusters from dark blue (low), light blue to white (medium), and red (high).

Cluster	1	2	3	4	5	6	7
1		0.151	0.207	0.069	0.704	0.445	0.738
2			0.103	0.179	0.686	0.372	0.724
3				0.235	0.672	0.302	0.713
4					0.706	0.460	0.739
5						0.731	0.560
6							0.714

Scans for selective sweeps

Tajima's D statistic was used to assess possible genomic sweeps associated with selection for each cluster formed. Genomic regions with low or negative Tajima's D values indicate an unusually high number of high-frequency variants due to a balanced selection. On the other hand, high positive values are due to an excess of rare variants which can be result of a positive selection (Tajima, 1989). Cluster 1 displayed the highest weighed mean Tajima's D value (0.854), while cluster 5 presented the lowest value (0.356) (Supplementary Data: Figure 2).

Clusters 1 to 4, composed mostly by cultivated *C. annuum* accessions presented several regions with positive Tajima's D values indicating a positive selection, possibly related to domestication and/or the pressure to achieve a specific phenotype resulting in an accumulation of trait related mutations (Nicolaï et al., 2013; Nimmakayala et al., 2016a, 2016b). High Tajima's D values for a region spanning 7.5Mb in the final part of the chromosome 1 for clusters 1 to 4 was found. QTLs implied in fruit weight, length and diameter, and pedicel length were described for this region so this could be an indication of selection for such traits (Barchi et al., 2009; Han et al., 2016; Hill et al., 2017). Chromosome 5 showed a possibly purified region in the last positions for clusters 1 and 4, spanning 6 Mb and possibly linked to fruit diameter (Hill et al., 2017; Yarnes et al., 2013). Finally, Clusters 2 to 4 showed high Tajima's D values for a 1Mb region at the end of chromosome 6. This region is linked to the control of several fruit traits such as weight, diameter, pericarp thickness, length and shape (Chaim et al., 2003; Hill et al., 2017; Rao et al., 2003; Yarnes et al., 2013). Bear in mind that the resolution is insufficient to make strong assumptions however it sheds light into future association mapping studies.

Contrarily, clusters comprising only *C. annuum* wild ancestor *C. annuum var. glabriusculum* and closely related *C. chinense* and *C. frutescens* species presented a Tajima's D distribution closer to the neutral or balanced selection (Supplementary Data: Figure 2). This was expected due to the biological status of these accessions

(Supplementary Data: Table 1). They are not as exploited as the first four clusters and are often cultivated in open-pollination conditions (Hayano-Kanashiro et al., 2016; Moses and Umaharan, 2012). Therefore, rare alleles are maintained at low frequencies.

Capsicum baccatum cluster (Cluster 5) presented the lowest values, indicating a patron of none or little positive selection as F_{st} had predicted (Supplementary Data: Figure 2). Our data is in agreement with previous results by other authors (Albrecht et al., 2012; Ibiza et al., 2012). As mentioned before, *C. baccatum* is usually cultivated in isolated areas of South America which makes genetic exchanges rare and it has not been subjected to intensive breeding programs as *C. annuum*. Other works used this statistical tool to successfully detect genomic selective sweeps in *Capsicum* spp. Taitano et al. (2018) reported a purified region on chromosome 6 possibly due to positive selection that confers the phenotype of the Chile de Agua ecotype. Nimmakayala et al. (2016b) detected a positive selection for chromosome 4 of *C. baccatum* and low values for the other 11 chromosomes suggesting a neutral selection. Same authors, for *C. annuum*, observed positive values across the entire genome, except for chromosome 8 (Nimmakayala et al., 2016b).

Conclusions

Our study confirms the utility of genomic tools such as GBS in the identification of highly informative SNPs and its application in the study of the genetic relations between *Capsicum* germplasm accessions. Availability of these tools is of great relevance to *Capsicum* breeders. Here we explore the genetic diversity, genetic structure and genetic relationships of a collection of Spanish landraces and several foreign controls using a set of 4,083 genome-wide SNPs. Population structure seems to be defined mainly by geographic origin and fruit traits. Sweet bell-shaped, blocky or thick-fleshed Spanish landraces and other European varieties located separately from pungent small American accessions. *Capsicum annuum var. glabriusculum* seems to have two genetic pools, one closer to *C. annuum* and another closer to *C. chinense* and *C. frutescens*. *Capsicum annuum* accessions have a higher level of positive selection and purified genomic regions according to Tajima's D statistics and lower diversity among them. This study sheds light on the origin of Spanish landraces origins and their genetic structure and genetic relations, and provides important information for future association studies and for breeding programs that contribute to the enhancement and protection of these materials.

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Chapter III: Fruit nutritional content characterization in *Capsicum* spp.

Characterization of protein, ascorbic acid, and mineral composition in ají (*Capsicum baccatum* L.) and chili (*Capsicum annuum* L.) under two different cultivation systems as a first step towards selection of pre-breeding elite materials

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Introduction

Vegetables are a great source of health-promoting compounds, and help to reduce the risk of incidence of cancer and cardiovascular diseases when consumed regularly (Fiedor and Burda, 2014; Yahia et al., 2019). Regarding that, peppers (*Capsicum* spp.) emerge as one of the most important crops, providing carotenoids, phenols, fats, oils, and minerals, to several cuisines around the world (Bosland and Votava, 2012; DeWitt and Bosland, 2009; Morales-Soto et al., 2014; Pérez-López et al., 2007b; Ribes-Moya et al., 2018; Rodríguez-Burruezo et al., 2010).

Nowadays, there is a growing concern, not just to meet productivity standards, but also to provide tastier and nutritionally rich products in order to satisfy consumer's demands (Casals et al., 2011; Egea-Fernández et al., 2018; Hurtado et al., 2014). Regarding pepper, production is mainly based on F_1 hybrids from 'California Wonder', 'Lamuyo' or 'Dulce Italiano' types, which have displaced traditional materials despite their narrower genetic diversity and "lack of taste" (Parisi et al., 2017; Rivera et al., 2016). Fortunately, the increasing interest for the "taste of the past" from consumers and the challenge of adapting to climate change conditions are contributing to the improvement and reintroduction of ancient materials (Brugarolas et al., 2009; Rivera et al., 2016). Bearing that, exhaustive characterization of the internal content of the available germplasm is of paramount importance in order to assess the existing diversity and to provide breeders with fitter individuals to be used in breeding programs for improved content in bioactive compounds (Rodríguez-Burruezo et al., 2005; Zonneveld et al., 2015).

On that matter, minerals and vitamins are small molecules with a major role in the human body (Sarpras et al., 2019; Yahia et al., 2019). Macrominerals, such as phosphorus and potassium, are required in greater doses than 100 mg/daily, whereas microminerals, such as iron and zinc, are needed in much smaller dosage, in order to provide a balanced diet (Institute of Medicine, 2019). Aside from minerals, vitamins are also necessary for a healthy diet (Carr and Maggini, 2017). On that matter, *Capsicum* fruits provide essentially vitamins A, C and E, although most of pepper's high antioxidant activity is due to vitamin C, also known as L-ascorbic acid (Chu et al., 2002). Hence, the recommended daily dose for ascorbic acid is around 90 mg/daily (Institute of Medicine, 2019). Furthermore, contrarily to other relevant health-promoting compounds, such as carotenoids and phenols (Parisi et al., 2017; Ribes-Moya et al., 2018; Wahyuni et al., 2011), pepper pods mineral content has only been studied for a reduced number of varieties and *C. annuum* concentrated most of those efforts (Pérez-López et al., 2007b; Rubio et al., 2002). Pepper's vitamin C content, on the other hand, has been heavily studied over the years, since it is one of the most powerful antioxidants available to the human diet (Carr and Maggini, 2017). Nonetheless it is still a relevant indicator of nutritional quality (Ribes-Moya et al., 2018; Rodríguez-Burruezo et al.,

2013, 2009). We believe that the study of the mineral and ascorbic acid profile of a wider fraction of *Capsicum* germplasm would provide researchers information of which materials may be fit to use in breeding programs for improved fruit internal content or to be introduced directly into the market as high quality varieties.

Capsicum baccatum L., also known as *ají*, is one of the five domesticated species of the *Capsicum* genus and it has a major role in Andean gastronomic heritage (APEGA et al., 2009; Ugás and Mendonza, 2012). Despite that, only recently we have been seeing an expansion of its popularity outside South America, probably due to an increase of immigrant population in Europe, and to the interest in ethnic food by the Europeans (Rodríguez-Burruezo et al., 2009; Ugás and Mendonza, 2012). In addition, this species represents a remarkable genetic pool for several traits of interest and is an asset of unexplored variability and richness in bioactive compounds (Rodríguez-Burruezo et al., 2009; Scaldaferro et al., 2018; Yoon et al., 2006; Zonneveld et al., 2015). In order to respond to the increasing demand for these varieties outside their natural habitat, it is of paramount importance, first, the creation of a breeding program in order to improve the adaptation of these materials to the Mediterranean region and, second, to improve their internal content in order to provide the consumer with a quality product at the same time that guarantees the success of the varieties among producers and consumers (Parisi et al., 2017; Rodríguez-Burruezo et al., 2009). This work represents a first step towards the understanding of how mineral and antioxidant content of a group of landraces of *C. baccatum* respond under Mediterranean conditions and two cultivation systems.

Material and methods

Plant material

Sixteen *C. baccatum* traditional varieties were considered herein and compared against three, well characterized, *C. annuum* traditional varieties as controls (Rodríguez-Burruezo et al., 2009) (Table 1). These materials belong to the COMAV *Capsicum* breeding group and are conserved at COMAV germplasm bank (Universitat Politècnica de València). *Capsicum baccatum* accessions were selected because they encompass a comprehensive variety of places of origin, morphological traits, and levels of other relevant bioactive compounds, such as carotenoids and phenolics (Rodríguez-Burruezo et al., 2009).

Table 1 – List of accessions and their corresponding species, origin, and fruit colour at ripening stage for the 19 cultivars evaluated in this work.

Accession	Species	Origin	Ripe Colour
11R	*Capsicum baccatum*	Cochabamba, Bolivia	Red
11O	*Capsicum baccatum*	Cochabamba, Bolivia	Orange
37R	*Capsicum baccatum*	Chuquisaca, Bolivia	Red
37O	*Capsicum baccatum*	Chuquisaca, Bolivia	Orange
58	*Capsicum baccatum*	Cochabamba, Bolivia	Red
106	*Capsicum baccatum*	Santa Cruz, Bolivia	Orange
120	*Capsicum baccatum*	Santa Cruz, Bolivia	Red
122	*Capsicum baccatum*	Santa Cruz, Bolivia	Red
127	*Capsicum baccatum*	Santa Cruz, Bolivia	Yellow
134	*Capsicum baccatum*	Santa Cruz, Bolivia	Red
140	*Capsicum baccatum*	Santa Cruz, Bolivia	Red
149	*Capsicum baccatum*	Santa Cruz, Bolivia	Orange
155	*Capsicum baccatum*	Santa Cruz, Bolivia	Red
165	*Capsicum baccatum*	Santa Cruz, Bolivia	Red
175	*Capsicum baccatum*	Chuquisaca, Bolivia	Red
178	*Capsicum baccatum*	Chuquisaca, Bolivia	Red
Ancho	*Capsicum annuum*	USA	Red
Guindilla	*Capsicum annuum*	Spain	Red
Pasilla	*Capsicum annuum*	Mexico	Brown

Cultivation conditions

Sterile seeds were put to germinate in petri dishes, containing a wet layer of cotton under a filter paper disk, under climate chamber conditions until two-cotyledon stage. Germinated seeds were transferred to seedling trays, filled with Neuhaus N3 substrate (Klasmann-Dellmann GmbH, Geeste, Germany), and kept under heated nursery greenhouse conditions till five-leaves stage. Finally, four seedlings per cultivar and cultivation system were transplanted to the greenhouse (GH) and open field (OF) plots at COMAV experimental fields (Universitat Politècnica de València Vera Campus GPS coordinates: 39°28'56.33"N; 0°20'10.88"W). Experimental design followed a completely randomized plot with 0.4 m and 1 m, within and between rows, respectively, in both cultivation systems. Drip irrigation system was used to provide water and fertilizer (1 g/L of commercial fertilizer $15N\text{-}5P_2O_5\text{-}30K_2O$). In addition, treatment against *Tetranychus urticae* and *Bemisia tabaci* was performed when necessary and depending on population levels, with greater incidence in our open field plot. Individual plants were trained with vertical strings and pruned accordingly to the standard local practices for pepper.

Sample preparation and analytical methods

Twenty ripe fruits per replica and cultivation system were harvested in each environment and split into two bulks. Half of fruits were washed, weighed (W, g), cut and dried until constant weight in a Raypa ID-150 oven (R. Espinar S.L., Barcelona, Spain) at 70ºC. Dried fruits were then powdered with a mechanical Taurus coffee grinder (Taurus Group, Oliana, Spain) and used to measure protein, dry matter and mineral content. Furthermore, the other half of the fruits was chopped and 10 g of fresh fruit tissue blended in 100 mL of sterile distilled water to determine ascorbic acid content.

L-ascorbic acid content (AA, mg/100g FW), was determined by potentiometric titration by selective electrode, using a Titrino 702 (Metrohm, Switzerland) and a 0.05 M chloramine T solution as standard. Dry matter (DM, g) was obtained using the formula 100 × [dry weight (DW, g)/fresh weight (FW, g)]. In addition, Nitrogen (N) content was determined through Kjeldahl method, using a Kjeltec 2100 distillation unit (Foss Tecator, Sweden) as N × 6.25, in order to estimate Protein percentage (PRT, % of DW). Finally, mineral content was determined on 2 g of dried samples calcined in a muffle at 450ºC for two hours. Light grey ashes were then weighted and dissolved in 2 ml of concentrated HCl (Scharlau, Valencia, Spain). The mixture was heated in a hot plate and mixed with 2 ml of distilled water, until first vapors appeared. Finally, the mixture was filtered with Whatman filter paper (Sigma-Aldrich, St. Louis, Missouri, USA) and the extract brought to 100 ml of distilled water (MAPA, 1994). On one hand, Phosphorus (P, mg/100g DW) content was determined by molibdovanadate method, using a Jenway 6305 UV-VIS (Jenway, UK) spectrophotometer. Potassium (K, mg/100g DW) and Sodium (Na, mg/100g DW) concentrations were obtained by flame photometry using a Jenway PFP7 (Jenway, UK). Ultimately, Magnesium (Mg, mg/100g DW), Iron (Fe, mg/100g DW) and Zinc (Zn, mg/100g DW) were estimated by atomic absorption spectrophotometry using a Thermo Elemental (Solaar AA Spectrometers, UK) (MAPA, 1994).

Statistical analysis

Multi-factorial analysis of variance (ANOVA) was performed using individual plant values to assess the genotype, environment, their interaction and residual effects (Hills and Jackson, 1978). In addition, Student-Newman-Keuls post-hoc multiple range test was performed to detect significant differences among accessions mean values. In addition, replica × environment interaction was studied by means of regression analysis using each replica mean values over the environment's mean values (Dabholkar, 1992). All statistical analysis were performed with Statgraphics Centurion XVII (StatPoint Technologies, Warrenton, VA, USA).

Results

A considerable diversity was found within our collection. Accession effect was highly significant (P<0.001 and P<0.01) for all evaluated traits. Likewise, the environment effect was highly significant (P<0.001) for most traits, with exception being K and Mg, for which no significant differences were detected, and Ca for which the environment effect was significant at a lower confidence level (P<0.05). In addition, interaction between those effects was significant for all traits. Finally, accession effect showed higher influence on W, K and Mg, whereas AA, PRT, P, Ca, Fe, and Zn were more dependent on the environment effect (Table 2).

Table 2 – Two-way ANOVA mean square values for accession (A), environment (E), accession × environment (A × E), and residual (R) effects for fruit weight (W, g), ascorbic acid (AA, mg/100g FW), protein content (PRT, % of DW), phosphorus (P, mg/100g DW), potassium (K, mg/100g DW), magnesium (Mg, mg/100g DW), calcium (Ca, mg/100g DW), iron (Fe, mg/100g DW), and zinc (Zn, mg/100g DW).

Effects	Df [1]	W [2]	AA	PRT	P	K	Mg	Ca	Fe	Zn
A	18	1975***	24206***	13.38***	23168***	1831040***	1211***	4496**	1.28**	14.12**
E	1	232***	89527***	68.51***	117594***	602561 ns	276 ns	9227*	13.97***	202***
AxE	18	30.43*	2684***	3.57***	10535***	898787***	1006***	6407***	1.86***	13.69**
R	150	17.22	392	0.88	2185	214423	302	2136	0.53	6.10

[1] Degrees of freedom.

[2] Mean square values. ***, **, * and ns indicate significant at P<0.001, P<0.01, P<0.05 or non-significant, respectively.

Regarding fruit weight (W), values ranged between 3.14 g (106) and 58.42 g (Ancho), under greenhouse (GH) conditions, and from 2.52 g (165) to 67.17 g (Ancho) under open-field (OF) conditions. It averaged 13.49 g (GH) and 10.95 g (OF) under our experimental conditions, where *C. annuum* accessions showed a much higher W than *C. baccatum*. In addition, *C. annuum* was stable between environments, regarding W (Table 3). Furthermore, ascorbic acid (AA) levels ranged from 35.50 mg/100g FW (37O) to 168.46 mg/100g FW (Guindilla) and between 52.32 mg/100g FW (106) and 250.53 mg/100g FW (Guindilla) under GH and OF conditions, respectively. Under GH the levels of this compound averaged 65.50 mg/100g FW, while under OF our collection averaged 110.25 mg/100g FW. In addition, *C. annuum* presented significantly higher values than the ones showed by *C. baccatum* accessions (Table 3). Finally, protein content (PRT) values ranged from 5.98% to 10.89% (37O and 11O, respectively) under GH conditions and between 7.21% (11R) and 12.01% (Pasilla) when cultivated under OF conditions. Protein content (PRT) slightly increased under OF cultivation, 9.09% against 7.84% under GH, and was slightly higher in *C. annuum* fruits (Table 3).

Table 3 – Accession, species and global mean values and standard deviation for weight (W), ascorbic acid (AA) and protein content (PRT) under Greenhouse (GH) and Open Field (OF) conditions. *F* statistic value indicates if there are significant differences between cultivation systems.

	Fruit weight (W, g)					Ascorbic acid (AA, mg/100g FW)					Protein content (PRT, % of DW)				
Accession	**GH**		**OF**		***F*[2]**	**GH**		**OF**		***F***	**GH**		**OF**		***F***
11O	5.62 ± 0.56	a-d[1]	3.38 ± 0.88	a	0.73 ns	48.46 ± 10.31	a	91.12 ± 24.38	ab	11.61***	10.88 ± 0.60	h	9.70 ± 1.14	b-d	3.98*
11R	4.75 ± 0.21	a-c	3.79 ± 0.80	a	0.13 ns	43.59 ± 7.70	a	82.82 ± 9.49	ab	9.82**	7.81 ± 0.58	c-f	7.21 ± 0.49	a	1.02 ns
37O	28.23 ± 0.89	f	26.58 ± 1.80	c	0.39 ns	35.50 ± 3.98	a	98.61 ± 16.82	ab	25.40***	5.97 ± 0.19	a	7.99 ± 0.45	a-c	11.56***
37R	26.48 ± 1.93	f	18.54 ± 4.64	b	9.16**	42.16 ± 6.24	a	120.32 ± 23.87	b	38.95***	8.11 ± 0.87	d-g	8.47 ± 0.83	a-d	0.36 ns
58	8.23 ± 1.43	a-d	5.81 ± 0.70	a	0.85 ns	85.44 ± 4.28	b	100.21 ± 18.71	ab	1.39 ns	8.21 ± 0.70	e-g	10.12 ± 2.17	cd	10.30**
106	3.13 ± 0.30	a	3.01 ± 0.49	a	0.00 ns	38.66 ± 0.93	a	52.32 ± 17.14	a	1.19 ns	7.77 ± 0.39	c-f	9.80 ± 1.91	cd	11.78***
120	4.19 ± 1.85	ab	3.13 ± 0.69	a	0.16 ns	56.89 ± 2.30	a	60.45 ± 12.98	a	0.08 ns	6.79 ± 0.24	a-e	8.88 ± 1.04	a-d	12.49***
122	8.32 ± 0.63	a-d	4.63 ± 1.89	a	1.97 ns	43.20 ± 8.97	a	66.23 ± 19.13	a	3.38 ns	7.67 ± 0.51	b-f	7.73 ± 0.62	ab	0.01 ns
127	3.17 ± 0.39	a	2.59 ± 0.44	a	0.05 ns	47.49 ± 4.30	a	84.86 ± 11.35	ab	8.90**	8.62 ± 0.88	fg	10.19 ± 0.97	d	6.69**
134	13.31 ± 0.80	de	8.00 ± 3.91	a	4.10*	58.63 ± 8.86	a	87.34 ± 9.90	ab	5.26*	6.29 ± 0.25	ab	8.16 ± 0.29	a-d	9.95**
140	16.73 ± 0.98	e	8.26 ± 4.22	a	10.43**	52.31 ± 12.23	a	74.09 ± 7.28	a	3.02 ns	7.18 ± 0.87	a-e	8.88 ± 1.28	a-d	8.24**
149	5.01 ± 0.88	a-c	4.43 ± 0.85	a	0.05 ns	37.46 ± 1.12	a	66.85 ± 6.59	a	5.51*	8.78 ± 0.63	fg	9.92 ± 0.65	cd	3.70 ns
155	5.15 ± 0.49	a-c	4.23 ± 0.71	a	0.12 ns	48.09 ± 6.15	a	59.77 ± 21.60	a	0.87 ns	6.51 ± 0.46	a-c	9.93 ± 0.73	cd	33.20***
165	4.47 ± 0.66	ab	2.52 ± 0.80	a	0.56 ns	45.43 ± 8.10	a	66.29 ± 12.48	a	2.77 ns	6.80 ± 0.66	a-e	10.05 ± 1.11	cd	30.05***
175	8.57 ± 3.43	a-d	6.12 ± 2.39	a	0.87 ns	90.35 ± 20.02	b	164.04 ± 18.23	c	34.64***	6.01 ± 0.37	a	7.55 ± 0.85	a	6.77*
178	12.90 ± 1.60	c-e	8.18 ± 2.78	a	3.24 ns	45.32 ± 17.34	a	84.32 ± 15.98	ab	9.70**	8.68 ± 1.17	fg	8.34 ± 0.52	a-d	0.33 ns
Ancho	58.42 ± 14.10	g	67.17 ± 16.85	d	11.10**	137.94 ± 12.13	d	231.28 ± 31.63	d	55.57***	6.73 ± 0.30	a-d	7.65 ± 1.17	a	2.38 ns
Guindilla	11.93 ± 1.96	b-e	7.94 ± 1.67	a	2.30 ns	168.46 ± 17.10	e	250.53 ± 9.78	d	42.96***	9.38 ± 0.49	g	9.72 ± 1.43	b-d	0.32 ns
Pasilla	27.70 ± 3.47	f	24.86 ± 2.37	c	1.17 ns	119.16 ± 1.61	c	249.38 ± 73.72	d	108.14***	10.68 ± 1.03	h	12.00 ± 0.52	e	4.99*
C. baccatum	9.89 ± 7.77		6.66 ± 6.08			51.19 ± 17.32		84.69 ± 30.90			7.63 ± 1.37		8.95 ± 1.41		
C. annuum	32.68 ± 21.57		33.32 ± 27.25			141.85 ± 23.89		243.73 ± 44.76			8.93 ± 1.82		9.79 ± 2.11		
Global mean	13.49 ± 13.75		10.95 ± 15.54			65.50 ± 37.99		110.25 ± 67.45			7.84 ± 1.52		9.09 ± 1.56		

[1] Different letters indicate significant mean differences among accessions within cultivation system, according to Student-Newman-Keuls post-hoc test for P<0.05.

[2] ***, **, * and ns indicate significant at P<0.001, P<0.01, P<0.05 or non-significant, respectively, differences found for a particular accession between cultivation systems.

Regarding macrominerals concentration values, phosphorus (P) presented values between 95.40 mg/100g DW (127) and 397.29 mg/100g DW (Guindilla) under GH conditions, and between 81.03 mg/100g DW (149) and 244.89 mg/100g DW (Pasilla), under OF conditions (Table 4). P content in fruits was slightly lower under OF conditions (125.57 mg/100g DW, on average) than under GH (177.18 mg/100g DW, on average). Furthermore, P concentration was, on average, higher in *C. annuum* fruits than in *C. baccatum*, although there were *C. baccatum* accessions that accumulated more P than some *C. annuum* (Table 4). Potassium (K) levels ranged from 1509.95 mg/100g DW (134) to 3977.39 mg/100g DW (Guindilla) under GH conditions, and from 1627.07 mg/100g DW (Ancho) to 3448.94 mg/100g DW (11O) under OF conditions (Table 4). K concentrations showed to be slightly lower when accessions were grown under OF conditions (2530 mg/100g DW, on average), compared to GH average (2652.21 mg/100g DW), although it was not statistically significant. *Capsicum baccatum* accessions presented a higher global average K concentration than *C. annuum* under both GH and OF conditions (Table 4). Regarding magnesium (Mg) our collection showed values ranging from 26.98 mg/100g DW (165) to 76.55 mg/100g DW (11R) under GH conditions, whereas OF values ranged from 18.94 mg/100g DW (149) to 81.55 mg/100g DW (37O). Mg global average concentration was slightly higher under OF conditions, although differences were not statistically significant (Table 5). *Capsicum baccatum* and *C. annuum* showed similar average Mg concentration under GH conditions, whereas under OF *C. baccatum* mean was higher (Table 5). Finally, calcium (Ca) values ranged between 3.66 mg/100g DW (127) and 155.40 mg/100g DW (134) under GH, and 25.04 mg/100g DW (122) and 155.57 mg/100g DW (127), under OF conditions (Table 5). Both species reduced average Ca concentration under OF conditions (107.34 mg/100g DW), compared to GH (120.72 mg/100g DW). Thus, *C. annuum* accessions showed higher Ca concentration than *C. baccatum* accessions under GH conditions, however, under OF conditions average concentrations were practically identical (Table 5).

Table 4 – Accession, species and global mean values and standard deviation for phosphorus (P) and potassium (K) under Greenhouse (GH) and Open Field (OF) conditions. *F* statistic value indicates if there are significant differences between cultivation systems.

	Phosphorus (P, mg/100 g DW)					Potassium (K, mg/100g DW)				
Accession	**GH**		**OF**		***F*[2]**	**GH**		**OF**		***F***
11O	183.97 ± 56.19	ab[1]	113.52 ± 22.34	ab	5.68*	3530.53 ± 416.08	d-f	3448.94 ± 306.86	c	0.08 ns
11R	199.87 ± 62.12	ab	87.65 ± 12.30	a	14.41***	2954.10 ± 467.25	c-e	2192.59 ± 96.86	ab	6.76*
37O	222.47 ± 22.33	ab	157.45 ± 6.76	ab	4.84*	2451.24 ± 216.43	bc	2866.66 ± 256.79	bc	2.01 ns
37R	124.25 ± 55.48	a	131.78 ± 51.54	ab	0.06 ns	2189.98 ± 593.44	a-c	2351.25 ± 390.44	ab	0.30 ns
58	205.07 ± 42.60	ab	176.94 ± 35.01	b	0.91 ns	2448.45 ± 303.23	bc	2568.69 ± 189.36	bc	0.17 ns
106	187.35 ± 48.19	ab	122.27 ± 47.78	ab	4.85*	2501.76 ± 140.83	bc	3049.35 ± 311.89	bc	3.50 ns
120	103.87 ± 27.18	a	97.69 ± 23.99	ab	0.04 ns	3080.50 ± 166.50	c-e	2753.24 ± 488.85	bc	1.25 ns
122	284.09 ± 83.60	b	113.15 ± 31.90	ab	33.43***	3650.87 ± 483.28	ef	2945.85 ± 403.88	bc	5.80*
127	95.40 ± 38.87	a	94.34 ± 35.62	ab	0.00 ns	2826.67 ± 720.02	c-e	2482.59 ± 587.28	ab	1.38 ns
134	117.80 ± 52.87	a	113.17 ± 32.22	ab	0.02 ns	1509.95 ± 303.95	a	2788.56 ± 409.22	bc	19.06***
140	176.45 ± 71.39	ab	127.39 ± 60.23	ab	2.75 ns	2610.61 ± 556.21	b-d	2094.15 ± 795.27	ab	3.11 ns
149	99.07 ± 42.20	a	81.03 ± 29.74	a	0.37 ns	2809.48 ± 337.08	c-e	2535.70 ± 238.90	ab	0.87 ns
155	102.07 ± 45.07	a	117.36 ± 20.25	ab	0.27 ns	2403.95 ± 395.06	bc	2384.93 ± 387.00	ab	0.00 ns
165	115.48 ± 43.18	a	98.47 ± 30.28	ab	0.33 ns	2473.75 ± 399.60	bc	2610.45 ± 421.88	bc	0.22 ns
175	147.73 ± 34.16	a	111.38 ± 35.96	ab	1.51 ns	2583.26 ± 319.11	b-d	2752.11 ± 279.92	bc	0.33 ns
178	194.08 ± 27.58	ab	145.80 ± 36.96	ab	2.67 ns	2882.91 ± 348.01	c-e	2187.11 ± 389.97	ab	5.64*
Ancho	185.32 ± 57.48	ab	108.15 ± 61.96	ab	6.81***	1715.25 ± 284.45	ab	1627.07 ± 778.68	a	0.09 ns
Guindilla	397.29 ± 70.74	c	154.09 ± 83.39	ab	67.68***	3977.38 ± 851.93	f	2379.11 ± 1030.23	ab	29.78***
Pasilla	224.84 ± 88.75	ab	244.89 ± 46.01	c	0.46 ns	1791.32 ± 69.76	ab	2178.05 ± 213.52	ab	1.74 ns
C. baccatum	159.94 ± 68.94		117.25 ± 40.45			2681.75 ± 612.71		2620.63 ± 510.77		
C. annuum	269.15 ± 116.85		169.04 ± 84.94			2494.65 ± 1192.30		2061.41 ± 781.62		
Global mean	177.18 ± 87.19		125.57 ± 53.30			2652.21 ± 727.02		2530.76 ± 595.57		

[1] Different letters indicate significant mean differences among accessions within cultivation system, according to Student-Newman-Keuls post-hoc test for P<0.05.
[2] ***, **, * and ns indicate significant at P<0.001, P<0.01, P<0.05 or non-significant, respectively, differences found for a particular accession between cultivation systems.

Finally, regarding microminerals concentration, iron (Fe) showed values between 0.55 mg/100g DW (149) and 3.05 mg/100g DW (Guindilla) and between 1.20 mg/100g DW (Ancho) and 2.79 mg/100g DW (Pasilla) regarding GH and OF conditions, respectively (Table 6). Global means indicate an increase of Fe under OF, although it was not significant. Interestingly, *C. baccatum* averaged higher under OF conditions whereas *C. annuum* accessions showed higher Fe concentration under GH conditions (Table 6). Finally, Zinc (Zn) concentrations showed remarkable high levels of variation, particularly under OF conditions, even within accession replicas. Hence, Zn ranged from 0.05 mg/100g DW (120) and 1.18 mg/100g DW (Ancho) under GH conditions, and from 0.49 mg/100g DW (11O) up to 7.50 mg/100g DW (Pasilla) when cultivated on OF conditions (Table 6). OF conditions (2.75 mg/100g DW) lead to a higher average concentration of this mineral, compared to GH conditions. Regarding species, *C. annuum* presented considerable higher values under both environments (Table 6).

Table 5 – Accession, species and global mean values and standard deviation for magnesium (Mg) and calcium (Ca) under Greenhouse (GH) and Open Field (OF) conditions. *F* statistic value indicates if there are significant differences between cultivation systems.

	Magnesium (Mg, mg/100g DW)					Calcium (Ca, mg/100 g DW)				
Accession	**GH**		**OF**		***F*[2]**	**GH**		**OF**		***F***
11O	74.07 ± 17.80	bc[1]	51.78 ± 10.02	a-e	4.11*	153.06 ± 43.67	b	148.48 ± 39.51	b	0.02 ns
11R	76.55 ± 15.65	c	41.34 ± 5.98	a-d	10.26**	141.51 ± 46.08	b	133.72 ± 51.53	b	0.07 ns
37O	53.08 ± 9.14	a-c	81.55 ± 9.42	e	6.71 ns	125.46 ± 23.53	b	54.84 ± 4.25	ab	5.84*
37R	63.28 ± 27.48	a-c	65.71 ± 33.04	de	0.05 ns	102.99 ± 28.67	b	112.31 ± 46.88	ab	0.10 ns
58	50.25 ± 25.12	a-c	52.67 ± 11.09	a-e	0.05 ns	106.10 ± 23.64	b	90.75 ± 63.75	ab	0.28 ns
106	35.93 ± 5.56	a-c	50.63 ± 20.29	a-e	1.79 ns	137.62 ± 53.83	b	73.03 ± 77.13	ab	4.88*
120	40.72 ± 13.63	a-c	38.95 ± 9.97	a-d	0.03 ns	128.07 ± 18.44	b	100.09 ± 86.87	ab	0.92 ns
122	55.21 ± 10.09	a-c	59.03 ± 21.82	b-e	0.12 ns	91.24 ± 18.59	b	25.04 ± 5.98	a	5.13*
127	43.30 ± 10.95	a-c	27.69 ± 13.52	a-c	2.02 ns	3.66 ± 0.93	a	155.57 ± 39.24	b	27.01***
134	43.83 ± 9.65	a-c	54.59 ± 23.60	b-e	0.96 ns	155.40 ± 65.83	b	91.24 ± 53.22	ab	4.82*
140	34.99 ± 15.57	a-c	45.21 ± 23.28	a-d	0.86 ns	119.77 ± 7.22	b	121.11 ± 34.44	ab	0.00 ns
149	32.20 ± 5.66	ab	18.94 ± 10.89	a	1.46 ns	99.20 ± 18.17	b	118.32 ± 37.17	ab	0.43 ns
155	30.02 ± 6.79	a	68.22 ± 18.42	de	12.08***	136.69 ± 10.62	b	89.72 ± 61.80	ab	2.58 ns
165	26.98 ± 7.44	a	47.24 ± 15.12	a-e	3.40 ns	95.59 ± 8.70	b	144.87 ± 59.98	b	2.84 ns
175	41.07 ± 17.01	a-c	53.99 ± 5.86	b-e	1.38 ns	138.28 ± 61.65	b	147.81 ± 7.90	b	0.11 ns
178	45.42 ± 18.00	a-c	54.65 ± 30.92	b-e	0.70 ns	146.41 ± 23.08	b	97.60 ± 85.36	ab	2.79 ns
Ancho	36.96 ± 17.90	a-c	24.21 ± 9.11	ab	1.35 ns	128.53 ± 9.66	b	103.13 ± 23.64	ab	0.76 ns
Guindilla	60.72 ± 31.83	a-c	26.02 ± 8.99	a-c	9.97**	136.55 ± 29.06	b	102.16 ± 46.72	ab	1.38 ns
Pasilla	31.89 ± 24.18	ab	61.14 ± 16.44	c-e	7.08**	147.58 ± 19.39	b	112.20 ± 59.58	ab	1.46 ns
C. baccatum	46.68 ± 19.39		50.11 ± 22.06			117.57 ± 46.73		107.63 ± 60.63		
C. annuum	43.19 ± 26.37		37.12 ± 20.82			137.55 ± 20.61		105.83 ± 43.27		
Global mean	46.13 ± 20.48		48.02 ± 22.29			120.72 ± 44.16		107.34 ± 58.03		

[1] Different letters indicate significant mean differences among accessions within cultivation system, according to Student-Newman-Keuls post-hoc test for P<0.05.

[2] ***, **, * and ns indicate significant at P<0.001, P<0.01, P<0.05 or non-significant, respectively, differences found for a particular accession between cultivation systems.

Table 6 – Accession, species and global mean values and standard deviation for iron (Fe) and zinc (Zn) under Greenhouse (GH) and Open Field (OF) conditions. *F* statistic value indicates if there are significant differences between cultivation systems.

	Iron (Fe, mg/100g DW)					Zinc (Zn, mg/100g DW)				
Accession	**GH**		**OF**		***F*** [2]	**GH**		**OF**		***F***
11O	1.28 ± 0.36	a-c[1]	1.36 ± 0.29	a	0.03 ns	0.54 ± 0.32	a-e	0.49 ± 0.23	a	0.00 ns
11R	1.33 ± 0.28	a-c	1.79 ± 0.54	a	1.00 ns	0.77 ± 0.24	b-e	0.58 ± 0.15	a	0.02 ns
37O	1.31 ± 0.32	a-c	2.57 ± 0.22	a	7.44**	0.95 ± 0.41	c-e	1.68 ± 0.15	a-c	0.22 ns
37R	1.22 ± 0.44	a-c	2.74 ± 1.54	a	10.87**	0.40 ± 0.19	a-d	0.89 ± 0.77	ab	0.10 ns
58	0.96 ± 0.22	a-c	1.83 ± 0.35	a	3.56 ns	0.78 ± 0.20	b-e	0.72 ± 0.26	ab	0.00 ns
106	1.34 ± 0.20	a-c	1.46 ± 0.83	a	0.07 ns	0.91 ± 0.34	b-e	1.40 ± 1.36	ab	0.10 ns
120	1.02 ± 0.17	a-c	1.23 ± 0.22	a	0.20 ns	0.05 ± 0.02	a	1.09 ± 1.69	ab	0.45 ns
122	1.62 ± 0.14	a-d	1.58 ± 0.60	a	0.00 ns	0.64 ± 0.17	a-e	1.27 ± 1.13	ab	0.17 ns
127	1.04 ± 0.20	a-c	2.14 ± 0.77	a	5.72*	0.50 ± 0.14	a-e	1.73 ± 1.85	a-c	0.62 ns
134	1.92 ± 0.89	cd	2.64 ± 1.41	a	2.42 ns	0.28 ± 0.16	a-d	5.55 ± 5.57	a-c	11.53***
140	0.73 ± 0.29	a-c	1.69 ± 0.45	a	4.32*	0.19 ± 0.21	ab	2.71 ± 3.10	a-c	2.64 ns
149	0.55 ± 0.12	a	2.36 ± 1.70	a	15.32***	0.22 ± 0.17	a-c	1.07 ± 0.57	ab	0.30 ns
155	0.84 ± 0.01	a-c	2.20 ± 0.18	a	8.79**	0.70 ± 0.05	a-e	2.03 ± 0.67	a-c	0.75 ns
165	0.65 ± 0.14	ab	1.99 ± 0.49	a	8.46**	0.21 ± 0.09	ab	7.14 ± 5.16	bc	19.98***
175	1.85 ± 1.17	b-d	2.10 ± 1.03	a	0.29 ns	0.62 ± 0.39	a-e	6.67 ± 5.70	a-c	15.21***
178	1.66 ± 0.55	a-d	1.85 ± 0.86	a	0.18 ns	0.86 ± 0.24	b-e	4.21 ± 4.45	a-c	4.67*
Ancho	2.57 ± 0.53	de	1.20 ± 0.68	a	8.83**	1.18 ± 0.38	e	4.31 ± 3.90	a-c	4.08*
Guindilla	3.05 ± 0.51	e	1.61 ± 0.86	a	9.82**	0.87 ± 0.40	b-e	0.93 ± 1.12	ab	0.00 ns
Pasilla	1.63 ± 0.80	a-d	2.79 ± 0.54	a	6.37*	1.01 ± 0.68	de	7.50 ± 5.92	c	17.54***
C. baccatum	1.21 ± 0.56		1.96 ± 0.91			0.54 ± 0.35		2.47 ± 3.42		
C. annuum	2.42 ± 0.84		1.87 ± 0.96			1.02 ± 0.48		4.25 ± 4.77		
Global mean	1.40 ± 0.75		1.94 ± 0.91			0.62 ± 0.41		2.75 ± 3.70		

[1] Different letters indicate significant mean differences among accessions within cultivation system, according to Student-Newman-Keuls post-hoc test for P<0.05.

[2] ***, **, * and ns indicate significant at P<0.001, P<0.01, P<0.05 or non-significant, respectively, differences found for a particular accession between cultivation systems.

Discussion

Several studies have shed light into the nutritional content of *Capsicum* pods. However, those efforts were not equally distributed, regarding species or compound of interest. On that matter, *C. annuum* has been the predominant species and has concentrated most of the research efforts (Ribes-Moya et al., 2018; Rodríguez-Burruezo et al., 2010; Wahyuni et al., 2011), whereas *C. baccatum* has only started to get attention in the recent years, despite its known relevancy in other pepper breeding aspects, such as resistance to biotic stress and genetic diversity source (Ahn et al., 2018; Rodríguez-Burruezo et al., 2009; Yoon et al., 2006). Likewise, phenols, volatile and carotenoids profiles have received considerably more attention than minerals (Olguín-Rojas et al., 2019; Parisi et al., 2017; Ribes-Moya et al., 2018; Wahyuni et al., 2011; Zonneveld et al., 2015).

With the increase of South American immigrant population in Europe and the increasing interest of the Europeans in ethnic food from that region of the globe, *C. baccatum* varieties are becoming increasingly demanded outside their natural region of cultivation and the opportunity to improve this species adaptation outside de Andean region has not been fully exploited yet (Rodríguez-Burruezo et al., 2009; Ugás and Mendonza, 2012). However, for any improved variety to succeed in the market it should provide both good productivity and good sensorial properties, in order to comply with the demands of both productors and consumers (Parisi et al., 2017; Rodríguez-Burruezo et al., 2009).

Herein, we aimed at studying the protein (PRT), ascorbic acid (AA) and mineral profiles, as well as their adaptation to Mediterranean cultivation conditions, of a set of *C. baccatum* accessions which showed interesting contents in other health-promoting compounds in a previous study under the same conditions tested here (Rodríguez-Burruezo et al., 2009). This work constitutes a first step towards the selection of individuals with most potential in order to be exploited in breeding programs for improved fruit quality and Mediterranean cultivation adaptation.

We observed great diversity for quality parameters among the collection, corroborating that *C. baccatum* is a diverse species (DeWitt and Bosland, 1996; Zonneveld et al., 2015). In addition, we observed a good adaptation of these materials to the Mediterranean conditions under both greenhouse and open-field conditions, with most genotypes yielding a similar amount of fruits as Guindilla, a Spanish *C. annuum* landrace (data not shown). This had already been reported by our research group in a previous trial (Rodríguez-Burruezo et al., 2009).

Regarding fruit weight (W), a great variation was observed among tested accessions, where *C. annuum* control accessions showed, on average, bigger fruits. During the domestication process, plants went through important physiological and metabolic changes resulting, for example, in increased fruit size (Pickersgill, 1997, 1971). On that

regard, *C. baccatum* has not underwent major breeding programs and is still traditionally cultivated in isolated areas and often maintained as semi-wild shrubs with small fruits, contrastingly to *C. annuum*, which may explain the differences of sizes (Ibiza et al., 2012; Scaldaferro et al., 2018). In addition, W was negatively affected when plants were cultivated in OF conditions, probably due to a higher incidence of abiotic stresses and to less available water due to evaporation.

Regarding internal content, accessions showed a wide range of variation depending on both the compound and the cultivation system. Ascorbic acid (AA) content was significantly higher, up to 2-fold in some cases, under OF conditions and for *C. annuum* accessions. Our values are similar to the ones found for *C. baccatum* and slightly higher for *C. annuum* in previous works (Pérez-López et al., 2007a; Ribes-Moya et al., 2018; Rodríguez-Burruezo et al., 2009). Like many other bioactive compounds, AA is linked to plant's protection against biotic and abiotic stresses, like UV radiation and heat-stress, so it is not unusual to find these conclusions (Smirnoff and Wheeler, 2000). In fact, this was already observed in other works (Ribes-Moya et al., 2018; Rodríguez-Burruezo et al., 2009).

Concerning protein (PRT) and mineral content, we observed a wide range of variation where both cultivation environment and genotype played important roles. In general, OF conditions provided higher concentrations of PRT, Mg, and Zn. Whereas P and K were, on average, higher under GH conditions. In addition, *C. annuum* accessions showed higher concentrations for most of the mentioned traits. In fact, *C. baccatum* accessions showed only a higher concentration than *C. annuum* for K, under both cultivation environments, and Mg, under OF conditions. Furthermore, our findings regarding K, Mg, Ca, Fe, and Zn are in agreement with other works (Pérez-López et al., 2007b; Sarpras et al., 2019). P levels, on the other hand, were higher in other works with pepper (Sarpras et al., 2019). Unfortunately, we did not find other pepper reports in order to compare our PRT values.

Bear in mind that *C. annuum* accessions included in this work as controls are highly adapted to the Mediterranean conditions, as result of several years of selection and breeding by our research group. Contrastingly, *C. baccatum* is mostly cultivated in the high mountainous areas of Bolivia and Peru and its cultivation outside those areas is within the first generations and, therefore, breeding for productivity is still needed (Ibiza et al., 2012; Rodríguez-Burruezo et al., 2009). Notwithstanding, these results provide an optimistic insight into the possibility of improving *C. baccatum* regarding yield and bioactive compounds content in the near future.

Finally, according to our findings, a serving of pepper (100g FW) cultivated under our GH conditions could provide around 74% of the recommended dietary allowance (RDA) for AA, 24% for P, 56% for K, 13% for Mg, 12% for Ca, 17% for Fe, 5% of RDA for Zn and finally 8% of vegetable protein (Institute of Medicine, 2019).

Considering our OF conditions, the same portion could provide around 119% of AA RDA, 17% for P, 53% for K, 15% for Mg, 11% for Ca, 24% for Fe, 23% for Zn and finally around 9% of protein (Institute of Medicine, 2019).

Conclusions

Our findings confirm the wide variation for bioactive compounds and mineral content within *Capsicum* and particularly within *C. baccatum*. Furthermore, these findings corroborate that cultivation environment is a major factor affecting nutritional content of pepper pods. Regarding that, the good performance of most of *C. baccatum* accessions shows that there are opportunities to breed and select *C. baccatum* materials adapted to the Mediterranean conditions and at the same time with interesting internal content, regarding ascorbic acid, protein and mineral content. Our results indicate that improving these varieties cultivation under OF conditions would significantly improve their fruit internal quality. In conclusion, this takes us to believe that a breeding program to improve this species fruit content in health-promoting compounds would be very efficient.

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Chapter IV: Adaptation to phosphorus low input conditions

Main pepper germplasm (*Capsicum* spp.) root adaptations to phosphorus low input conditions

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Introduction

Agriculture will face many challenges in the next generations, especially those related to food security and agricultural sustainability (Grafton et al., 2015; Jaggard et al., 2010). On one hand, intensive agriculture has a significant impact on the environment, contributing to soil erosion, soil salinization, eutrophication and contamination of water bodies, and biodiversity reduction (Russo, 2012). On the other hand, poor and undeveloped nation's agricultural systems need to be improved in order to cope with requirements of an increasing population and the impact of climate change consequences (Jaggard et al., 2010; Raza et al., 2019).

In both cases, one of the most critical resources involved is phosphorus (P), a non-organic mineral with a major role within the physiochemical processes of plants (Jones, 2012; Schnug and Haneklaus, 2016a). Since almost 40% of the world's arable land lacks of P or the soil properties to make it available for crops, P absence is a major constraint to food production all around the world (Cordell et al., 2009; Mogollón et al., 2018; Vance et al., 2003). Until now, application of P-enriched fertilizers has been the main strategy to face deficiency in soils, despite the severe contaminant emissions associated to its production (Cordell et al., 2009; Schnug and Haneklaus, 2016b; Tilman et al., 2002). In addition, only 15 to 40% of added P is uptaken by crops (Cordell et al., 2009; Lynch, 2007; Tilman et al., 2002), while the remaining ends up being washed down through the soil, contributing to eutrophication of water bodies (Fernández and Selma, 1998; Kauranne and Kemppainen, 2016). Furthermore, as costs of extraction increase and rock-phosphate reserves decline, P is becoming an extremely expensive resource that is already unaffordable in many regions of the globe (Mogollón et al., 2018). As demand for P-enriched fertilizers is going to increase in the next decades, the control for P supplies will be a source of conflicts (Cordell et al., 2009; Schnug and Haneklaus, 2016a). Therefore, there is a need for adapted varieties to low P inputs.

The response to P-starvation conditions has been extensively studied for a few model organisms and some economically important crops over the last decades (Fernandez and Rubio, 2015; Fita et al., 2012; Hammond et al., 2009; Li et al., 2009; Lynch and Brown, 2001). As a result, researchers have linked several root traits to a greater performance under low P conditions (Niu et al., 2013). Thus, the increment of root hairs number, enhanced phosphatases and organic acids production, root P transporters enhanced expression, and root cellular structure alteration, are adaptations expressed under P-starvation (Fernandez and Rubio, 2015; Fita et al., 2012, 2011; Hammond et al., 2009; Miguel et al., 2015). The exploitation of these adaptations could have a remarkable impact on the reduction of chemical fertilizers inputs in agricultural systems (Lynch, 2007; van de Wiel et al., 2016).

Peppers (*Capsicum* spp.) are one of the most relevant vegetables, grown in almost all temperate and tropical regions of the world (Bosland and Votava, 2012). FAO's last available data estimates that around 40 million tonnes of pepper are produced each year in almost 4 million hectares of dedicated land (FAO, 2019). Therefore, improving pepper for its uptake and use of P would significantly reduce the need for fertilizer applications in many countries of the world (Lynch, 2007; Tilman et al., 2002). Notwithstanding, the development of improved *Capsicum* varieties for low input conditions is a challenging goal and is conditioned by both the availability of genetic variability within *Capsicum* and the understanding of the mechanisms underlying the response. Regarding the first point, *Capsicum* spp., particularly *C. annuum* L., are remarkably diverse, as well as adapted to a wide range of environments and, therefore, tolerant to several abiotic stresses (DeWitt and Bosland, 1996; Hwang et al., 2005; Jing et al., 2016; Sahitya et al., 2019). However, pepper fundamentals regarding this subject have never been studied. We believe that an exhaustive characterization of pepper germplasm for its responses under low P input conditions is of paramount importance in order to recognize the variability within the genus, to enhance our understanding regarding the responses activated under such conditions, and finally, to link those responses to genomic regions controlling them. Herein, we established as main goal the characterization of the main root adaptations of pepper accessions to low P conditions as a first step towards the identification of elite individuals for future pepper breeding programs.

Material and methods

Germplasm

A collection of 25 pepper accessions, encompassing 22 *C. annuum*, 2 *C. chinense* and 1 *C. frutescens* accessions, comprising a wide range of variability for fruit shape, fruit pungency, fruit colour, biotic resistances, adaptation to a range of environments, were studied herein (Pereira-Dias et al., 2019) (Table 1). The considered collection belongs to the COMAV Germplasm Bank (Universitat Politècnica de València) and to the COMAV *Capsicum* breeding group, and was selected based on previous experiments, where an interesting performance and diversity for several relevant root and P-uptake traits was observed (Fita et al., 2013).

Table 1 - List of the 25 accessions and corresponding abbreviation, species, varietal status, origin, fruit shape, fruit taste, fruit colour, and trial year.

Abbreviation	Species	Accession	Origin	Fruit shape	Fruit taste	Fruit colour	Trial
	Traditional varieties						
fra_DLL	*Capsicum annuum*	Doux Long des Landes	France (INRA-GEVES, F. Jourdan)	Cayenne, long-sized	Sweet	Red	Trial 2
mex_096D	*Capsicum annuum*	Chile ancho Poblano	Mexico, Aguascalientes (UAA)	Triangular, Pochard's C4 type	Hot	Red	Trial 2
mex_103B	*Capsicum annuum*	Chile ancho Poblano	Mexico, Aguascalientes (UAA)	Triangular, Pochard's C4 type	Hot	Red	Trial 2
mex_pas	*Capsicum annuum*	Pasilla Bajío	Mexico	Cayenne, long-sized	Hot	Brown	Trial 1 and 2
mex_ng	*Capsicum annuum*	Numex Garnet	Mexico, Aguascalientes (UAA)	Elongated, Pochard's C2 type	Sweet	Red	Trial 2
mu_esp	*Capsicum annuum*	Jalapeno Espinalteco	Mexico/USA (P. W. Bosland)	Jalapeno	Hot	Red	Trial 1 and 2
sp_060	*Capsicum annuum*	Pimiento morrón de bola (BGV-60)	Spain, Zamora	Round, Pochard's F type	Sweet	Red	Trial 2
sp_11814	*Capsicum annuum*	Dulce tipo italiano (BGV-11814)	Spain, León	Elongated, Pochard's C2 type	Sweet	Red	Trial 2
sp_bola	*Capsicum annuum*	P.D.O. Pimentón Murcia	Spain, Murcia	Round, Pochard's N type	Sweet	Red	Trial 1 and 2
sp_lam	*Capsicum annuum*	Lamuyo	Spain, Valencia	Blocky, Pochard's B1 or B2 type	Sweet	Red	Trial 2
sp_piq	*Capsicum annuum*	P.D.O. Pimiento Piquillo de Lodosa	Spain, Navarra	Triangular, Pochard's C4 type	Sweet	Red	Trial 1 and 2
usa_chi	*Capsicum annuum*	Chimayó	USA, New Mexico (P. W. Bosland)	Blocky small-sized, Pochard's B4 type	Hot	Red	Trial 1
usa_conq	*Capsicum annuum*	Numex Conquistador	USA, New Mexico	Elongated, Pochard's C2 type	Sweet	Red	Trial 2
usa_jap	*Capsicum annuum*	Chile Japonés	USA, New Mexico	Cayenne, very short-sized	Hot	Red	Trial 2
usa_numex	*Capsicum annuum*	Numex X	USA, New Mexico	Elongated, Pochard's C2 type	Hot	Red	Trial 2
usa_sandia	*Capsicum annuum*	Numex Sandia (BGV-13293)	USA, New Mexico	Elongated, Pochard's C2 type	Hot	Red	Trial 2
	Experimental lines						
mex_scm	*Capsicum annuum*	Serrano Criollo de Morellos	Mexico	Serrano	Hot	Red	Trial 1 and 2
sp_cwr	*Capsicum annuum*	California Wonder	Spain, Valencia (COMAV)	Blocky, Pochard's A type	Sweet	Red	Trial 1
	Commercial hybrids (F1)						
sp_anc	*Capsicum annuum*	Ancares	Spain (Ramiro Arnedo)	Blocky, Pochard's B1 or B2 type	Sweet	Red	Trial 2
sp_cat	*Capsicum annuum*	California Wonder Catedral	Spain (Zeraim Ibérica)	Blocky, Pochard's A type	Sweet	Red	Trial 1
sp_lobo	*Capsicum annuum*	El Lobo	Spain (Zeraim Ibérica)	Blocky, Pochard's A type	Sweet	Red	Trial 2
sp_mel	*Capsicum annuum*	Melchor	Spain (Ramiro Arnedo)	Blocky, Pochard's A type	Sweet	Red	Trial 1
	Other Capsicums						
bol_037	*Capsicum chinense*	Bol-37R	Chuquisaca, Bolivia	Triangular, small-sized, thin flesh	Hot	Red	Trial 1
bol_144	*Capsicum baccatum*	Bol - 144	Bolivia, Santa Cruz	Cayenne, very short-sized	Hot	Red	Trial 1
eq_973	*Capsicum chinense*	ECU - 973	Equador, Napo	Triangular, small-sized, thin flesh	Hot	Red	Trial 1

Germination and cultivation conditions

Seeds were surface sterelised with a 30% NaClO solution (v:v) for five minutes, followed by rinsing with steril dionized water, and transfered to individual Petri dishes containing a wet layer of cotton under a filter paper disk. Two drops of 2% Tetramethylthiuram disulphide solution were added to each Petri dish in order to prevent fungal proliferation. Petri dishes were kept under germination chamber conditions until two-cotyledon stage. Seedlings were then transferred to seedling trays filled with Neuhaus N3 substrate (Klasmann-Dellmann GmbH, Geeste, Germany), kept under heated nursery conditions until five leaves stage, and finally transplanted to the greenhouse.

The experiment was carried out in two consecutive years. In the first year (from now on Trial 1), 12 accessions were trialled and the 5 most interesting genotypes were re-trialled in the second year (from now on Trial 2), against 14 new accessions (Table 1). In both trial years, plants were grown for 60 days under a mesh greenhouse, during the spring-summer cycle, at COMAV experimental fields (Universitat Politècnica de València Vera Campus GPS coordinates: 39°28'56.33"N; 0°20'10.88"W). Transplant was carried out in June and the experiment was terminated in August. Nine (Trial 1) and six (Trial 2) replicas, per accession and treatment, were grown in 10 L plastic pots filled with substrate made by mixing a part of soil with a part of sand (1:1), and arranged into a completely randomized design with six rows. Pots were spaced 1.2 m between rows and 0.40 m inside row and drip irrigation system provided water and nutrient solutions to plants. Individual plants were trained with vertical strings, according to standard local practices for pepper. Plants were not pruned during the experiment in order not to interfere with biomass yield. Likewise, phytosanitary treatments against whiteflies, spider mites, aphids and caterpillars were applied accordingly to population levels.

Plants were subjected to two treatments. On one hand, Control treatment was applied using a standard solution providing all elements. On the other hand, stress treatment (from now on NoP) was applied using similar solution to Control treatment except for P carrying ions, which were removed from formulation of the solution. Ion concentrations of both Control and NoP solutions, as well of irrigation water, can be consulted in Supp. Table 1.

Sample preparation

After the 60 days period, in both trials, plants were harvested for processing. Shoot and fruits were processed separately in order to assess effects of P deprivation on both tissues. Each tissue was put into individual paper bags and dried at 70ºC, until constant weight, in a Raypa ID-150 oven (R. Espinar S.L., Barcelona, Spain). At this point, shoot (SW, g) and fruit (FW, g) dry weights were determined and those tissues grinded into a thin

powder, using a domestic Taurus coffee grinder (Taurus Group, Oliana, Spain), for posterior mineral content analysis. Furthermore, all replicates' roots were separated from subtract by gently washing them with running tap water and processed separately from other tissues (Fita et al., 2007). This was made by hand, one root at a time (Figure 1).

For Trial 1, root hairs (Ø < 0.5 mm; bear in mind that this term does not correctly translate to the root anatomical definition of root hairs, however, herein is useful to differentiate between the evaluated root parts) were separated from lateral roots (Ø > 0.5 mm) and dried at 70ºC, in a Raypa ID-150 oven (R. Espinar S.L., Barcelona, Spain), in order to obtain root hairs dry weight (RHW, g) (Figure 1). Secondly, lateral roots (Ø > 0.5 mm) were scanned, using an Epson Expression 1640XL G650C scanner (Seiko Epson Corp., Japan), and resulting images were analysed by WinRIZHO™ Pro 2.3 software (Regent Instruments Inc., Canada). Lateral root total length (LRL, m), lateral root average diameter (LRAD, mm) and total length of lateral roots with diameter under ($LRL_{<1mm}$, m), and above ($LRL_{>1mm}$, m) 1 mm, were determined based on said images for each replicate included in the experiment. Finally, scanned lateral roots were dried till constant weight at 70ºC in a Raypa ID-150 oven (R. Espinar S.L., Barcelona, Spain) in order to obtain lateral roots dry weight (LRW, g) and grinded for posterior mineral content determination (Figure 1). From those measurements, we calculated several parameters in order to better characterize plants performance. Hence, for trial 1, total root dry weight (RW, g) was determined as the sum of RHW and LRW and, therefore, total biomass dry weight (BW, g) calculated as the sum of RW, SW and FW. In addition, root to shoot weight ratio (RSR) was calculated by dividing RW by SW, RHW%, as percentage of dry weight devoted to root hairs, was calculated by division of RHW by RW, and proportion of root length devoted to fine lateral roots (PLFR, %) as the ratio between $LRL_{<1mm}$ and LRL. Finally, lateral root specific length (LRSL, m/g) was calculated by dividing LRL by LRW.

For Trial 2, roots were entirely scanned (Figure 1), using an Epson Expression 1640XL G650C scanner (Seiko Epson Corp., Japan), and resulting images were analysed by WinRIZHO™ Pro 2.3 software (Regent Instruments Inc., Canada). In order to fully capture root's morphometrics, individual roots were properly spread over several transparent acetate sheets (Figure 1). Root total length (RTL, m), total root average diameter (TAD, mm), and total length of roots with diameter under ($RL_{<1mm}$, m) and above ($RL_{>1mm}$, m) 1 mm, were determined for each replicate. Bear in mind that within Trial 2 traits correspond to the whole root, not just lateral roots as in Trial 1 (Figure 1). Finally, scanned roots were dried until constant weight at 70ºC in a Raypa ID-150 oven (R. Espinar S.L., Barcelona, Spain) and grinded to powder. Root hairs dry weight, (RHW, g) and lateral roots dry weight (LRW, g) were determined, as in Trial 1. Likewise, total root weight (RW, g) was determined as the sum of RHW and LRW and total biomass weight (BW, g) as the sum of RW, SW and FW. In addition, root to shoot ratio (RSR) was calculated dividing RW by SW, HRW% by division of RHW by RW, and

proportion of root length devoted to fine lateral roots, that is including root hairs and roots below 1 mm (PFR, %) as the ratio between $RL_{<1mm}$ and RTL. Finally, root specific length (RSL, m/g) was calculated by dividing RTL by RW.

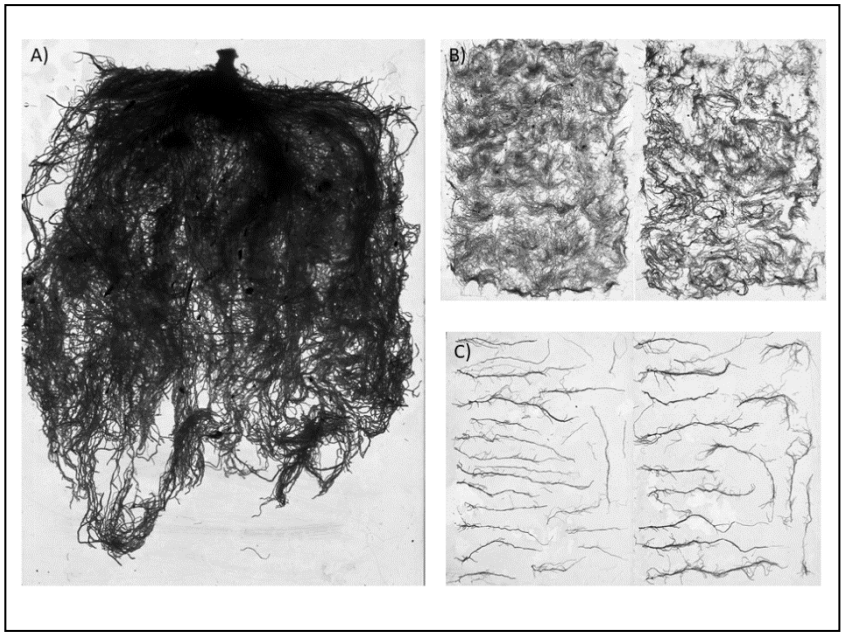

Figure 1 – Illustration of the roots along scanning process. Individual roots were separated from the soil with running tap water and taken to the laboratory to be scanned and dried. In Trial 1, whole roots (A) were separated into B) root hairs (Ø < 0.5 mm) and C) lateral roots (Ø < 0.5 mm). Root hairs (B) were only weighed while lateral roots (C) were scanned and weighed. In Trial 2, whole roots (A) were also separated into root hairs (B) and lateral roots (C) and both were scanned and weighed.

Tissue mineral concentration assessment

Before mineral content determination, samples were mineralized (MAPA, 1994). Thus, 2 g of powdered plant tissue were calcined for 2 hours in a muffle at 450ºC. Ashes resulting from mineralization were let to cool down, weighted, and then hydrated with 2 mL of distilled water followed by addition of 2 mL of concentrated HCl (Scharlau, Valencia, Spain). At this point, the solution was heated on a hot plate, until first fumes appeared, and then filtered with Whatman filter paper (Sigma-Aldrich, St. Louis, Missouri, USA). Finally, distilled water was added in order to make up to 100 mL volume (MAPA, 1994).

In Trial 1, concentration (g $100g^{-1}$ DW) of Phosphorus (P), Potassium (K), Calcium (Ca), Magnesium (Mg), Sodium (Na), and Sulphur (S) in different plant tissues (root, shoot and fruits, $[Mineral]_{Tissue}$) was determined by Inductively Coupled Plasma - Atomic Emission Spectrometry (ICP-AES; iCAp-AES 6000, Thermo Scientific, Cambridge UK). Samples were digested for 24 h by adding 10 mL 65% HNO_3 solution (Panreac Quimica S.A.U., Barcelona, Spain) to 0.5 g dried material, in a 25 mL open vessel. Digested samples were then boiled at 120ºC for 10 min followed by another 25 min at 170ºC. Finally, samples were cooled, added 2 mL of 70% $HClO_4$ (Panreac Quimica S.A.U., Barcelona, Spain), and put at 200ºC for 40 min. At this point, samples were transferred to a flask and volume was brought up to 25 mL with distilled water.

For Trial 2, leaves P-concentration ($[P]_{Shoot}$) was analysed by colorimetric reaction (MAPA, 1994). This method is based on absorbance measurement at 430 nm of each sample in acid solution and on the presence of vanadium (V^{5+}) and molybdenum (Mo^{6+}) ions. Under these conditions, phosphoric acid forms a phosphomolybvanadate complex that gives yellow coloration. Hence, 5 mL of mineralized solution were pipetted into a new 25 mL volumetric flask, followed by addition of 5 mL of nitro-vanado-molybdic reagent. Volume was then brought up to 25 mL with distilled water. Prior to mineral concentration determination, a standard curve was constructed with standards 0, 2, 4, 6, 8, 10 y 12 µg of P mL^{-1} prepared from an initial solution of 20 µg of P mL^{-1}. Sample P concentration was determined using a 6305 model UV/V spectrophotometer (Jenway, Gransmore Green, England, UK) at 430 nm against standard curve.

Phosphorus uptake and use efficiency parameters

In order to better characterize treatment effect on accessions performance, several widely-used P-uptake and P-use efficiency parameters (PUE) were calculated based on previous works (Fita et al., 2011; Hammond et al., 2009) (Table 2).

Table 2 - P-uptake and P-use efficiency parameters used in this experiment and corresponding abbreviation, formula and expressed units.

Parameter	Abbreviation	Formula	Units
Tissue P content	RootP, ShootP, FruitP	$[P]_{Tissue} \times DW_{Tissue}$	g
Plant total P content	PTP[1]	$[P]_{Root} \times DW_{Root} + [P]_{Shoot} \times DW_{Shoot} + [P]_{Fruit} \times DW_{Fruit}$	mg P
P uptake efficiency	PUpE[2]	$([P]_{Control} \times BW_{Control}) - ([P]_{NoP} \times BW_{NoP})$	mg P
P utilization efficiency	PUtE[2]	$(BW_{Control} - BW_{NoP}) / (([P]_{Control} \times BW_{Control}) - ([P]_{NoP} \times BW_{NoP}))$	g DW g^{-1} P
Physiological P use efficiency	PPUE	$BW_{Control}/[P]_{Control}$ and $BW_{NoP}/[P]_{NoP}$	g^2 DW g^{-1} P
P efficiency ratio	PER	$BW_{Control}/ ([P]_{Control} \times BW_{Control})$ and $BW_{NoP}/ ([P]_{NoP} \times BW_{NoP})$	g DW g^{-1} P

[1]Note that for Trial 2 only $[P]_{shoot}$ was measured, therefore PTP was obtained as $[P]_{shoot} \times BW$.
[2]Note that [P] in Trial 1 is the weighted average [P] among different tissues, whereas in Trial 2 [P] = $[P]_{shoot}$.

Statistical analysis

Two-way factorial analysis of variance (ANOVA) was performed using individual plant values in order to assess significance of both accession and treatment effects, the interaction between those effects, and the residuals (Hills and Jackson, 1978). We report herein the mean squares for those effects and corresponding residuals. In addition, Student-Newman-Keuls post-hoc multiple range test (P>0.05) was used to detect significant differences among accessions for each evaluated trait (Supp. Tables 3 and 4). Finally, multivariate Principal Component Analysis (PCA) was carried out using Euclidean pairwise distances and traits differences between treatments ($\mu_{Control} - \mu_{NoP}$). In addition, traits variation (%) when passing from Control to NoP conditions was calculated as $\left(\frac{\mu_{NoP} - \mu_{Control}}{\mu_{Control}}\right) \times 100\%$. All statistical analysis were performed with Statgraphics Centurion XVII (StatPoint Technologies, Warrenton, VA, USA) and plotted using R package ggplot2 (R Development Core Team, 2009; Wickham, 2016).

Results

Treatment effect on P and other minerals concentrations for Trial 1

P concentration [P] in plant tissues is an important indicator of both the treatment effectiveness and the accessions capability to make the most with the available resources. In Trial 1, plants cultivated under NoP conditions showed significantly lower P concentration compared to control plants. This behaviour was statistically significant for all three sampled tissues (Supp. Table 2). For $[P]_{Roots}$, we observed a reduction from 0.56 g P $100g^{-1}$ DW, when cultivated under Control conditions, to 0.10 g P $100g^{-1}$ DW (-81.76%), when cultivated under NoP conditions, where bol_037 (-91.62%) and sp_cwr (-90.53%) showed the biggest drop for this trait (Table 3). For $[P]_{Shoot}$, values decreased from 0.18 g $100g^{-1}$ DW to 0.12 g P $100g^{-1}$ DW (-29.31%) for Control and NoP conditions, respectively, and the most affected accessions were sp_piq (-45.26%) and sp_cwr (-45.03%) (Table 3). Finally, fruit P levels dropped from 0.26 g P $100g^{-1}$ DW, when irrigated with Control solution, to 0.17 g P $100g^{-1}$ DW (-35.18%), when NoP solution treatment was applied. Accessions sp_mel (-49.43%) and sp_cwr (-47.21%) showed the highest drop regarding $[P]_{Fruit}$ (Table 3).

Concentration of other macrominerals was determined in order to assess possible deficiencies induced by the applied treatments. In that regard, significant differences between treatments were observed for all minerals, with exceptions, depending on the tissue considered. In any case, the concentration for those minerals were within the normal range for pepper (Supp. Table 2).

Treatment effect on accumulated P and PUE parameters for Trial 1

Multi-factorial ANOVA (Table 3) was pursued in order to assess accession and treatment effects, as well as their interaction, for 22 different traits and parameters, encompassing P tissue concentration and accumulation traits (7), PUE parameters (4), and root and shoot morphometric traits (11).

As expected, treatment showed a significant effect on P accumulation in the different plant tissues as well as for the whole plant ($P<0.001$). In addition, a significant accession effect was also observed on ShootP, FruitP as well as on plant PTP ($P<0.001$), indicating differential response for P accumulation in tissues among accessions (Table 3). Interestingly, these differences were not significant for RootP. Finally, a significant interaction accession per treatment was observed for FruitP ($P<0.01$). Thus, our collection presented a wide variation for P tissue accumulation, where roots presented the biggest difference regarding P content between treatments (Figure 2).

On average, stressed plants had 86.16% less accumulated P in the root than Control plants. Thus, Bol-37r (-93.34%), sp_cwr (-92.86%), and usa_chi (-88.89%) were the accessions with the biggest drop in accumulated P between treatments (Table 4). Regarding ShootP, a significant reduction of P levels was observed, averaging 56.12% less than under Control conditions. Accessions mex_pas (-52.54%), mex_scm (-62.45%), mu_esp (-77.91%), sp_cwr (-55.30%), sp_piq (-59.93%), and usa_chi (-66.88%) presented significant drops. On that matter, mex_scm presented the biggest drop in ShootP levels (from 0.136 g to 0.051 g under Control and NoP treatment, respectively (Supp. Table 3)), despite that, this accession showed the highest P content of ShootP for both treatments (Figure 2 and Table 3). Likewise, FruitP content was negatively affected by stress treatment and plants had, on average, 34.32% less accumulated P in their fruits (Figure 2). Accessions sp_cat and usa_chi presented significant decrease of this mineral when cultivated under stress conditions; sp_cat (-78.90%) drop from 0.091 g to 0.019 g while usa_chi (-61.56%) went from 0.106 g to 0.041 g (Supp. Table 3), under Control and NoP, respectively (Figure 2 and Table 3). Finally, regarding PTP, genotypes showed a significant loss, averaging 63.30%, when cultivated under NoP and compared to Control replicas (Table 3). Accessions mu_esp (-76.17%), sp_cat (-73.96%) and sp_mel (-77.17%) presented the biggest difference between Control and NoP treatments (Figure 2). Detailed information about accessions performance can be found within Supp. Table 3.

Table 3 – Multi-factor ANOVA mean square values of accession (A) and treatment (T) effects, their interaction, and error (E) for P concentration traits $[P]_{Root}$, $[P]_{Shoot}$, $[P]_{Fruit}$, P content traits RootP (g P), ShootP (g P), FruitP (g P), PTP (mg P), and finally for efficiency parameters PPUE (g^2 DW g^{-1} P) and PER (g DW g^{-1} P).

Effect	$[P]_{Root}$	$[P]_{Shoot}$	$[P]_{Fruit}$	RootP	ShootP	FruitP	PTP	PPUE	PER
A	0.046* [a]	0.004**	0.010***	0.0001 ns	0.0027***	0.003***	5094**	5.26E08*	6.81E04**
T	5.080***	0.072***	0.194***	0.0189***	0.0251***	0.026***	217740***	1.16E09*	3.01E06***
A x T	0.045*	0.002 ns	0.002 ns	0.0001 ns	0.0007 ns	0.002*	1920 ns	3.04E08 ns	3.34E04 ns
E	0.019	0.001	0.001	0.0001	0.0004	0.0007	1827	2.12E+08	2.13E+04

[a] ***, **, * and ns indicate significant for $P<0.001$, $P<0.01$, $P<0.05$, and non-significant, respectively, obtained after analysis of variance (ANOVA) for individual accession values.

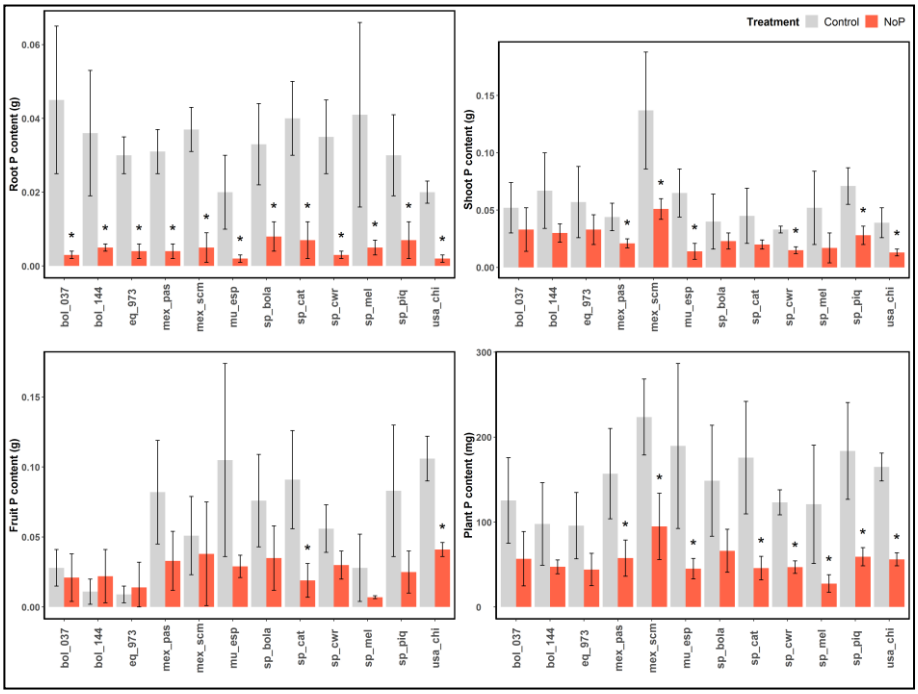

Figure 2 – RootP (g), ShootP (g), FruitP (g), and PTP (mg) values presented by accessions for both Control and NoP treatments for Trial 1. Asterisks indicate significant differences (P<0.05) between treatments for a given accession, whereas absence of asterisks indicates no significant differences between treatments were found. In addition, black bars indicate standard deviation for a particular accession and treatment.

In order to evaluate how efficient genotypes were under our experiment's conditions, we used widely extended parameters (Fita et al., 2011; Hammond et al., 2009). Hence, we estimated the Physiological P use efficiency (PPUE, g^2 DWg^{-1} P), P efficiency ratio (PER, g DW g^{-1} P), P uptake efficiency (PUpE, mg P), P utilization efficiency (PUtE, g DW g^{-1} P) to determine genotypes effectiveness uptaking and using P.

Thus, significant differences were found between treatments for these parameters. PPUE ($P<0.05$) and PER ($P<0.001$ and $P<0.001$, respectively) were significantly affected by both accession and treatment effects, with treatment being the most important effect (Table 3). No significant differences were detected for the interaction of effects for neither of the parameters (Table 3). Furthermore, PPUE was on average 36.26% higher under NoP conditions (Table 4). Under the Control treatment, values ranged between 14572 g^2 DW g^{-1} P (eq_973) and 41136 g^2 DW g^{-1} P (mex_pas), whereas under NoP conditions values ranged between 14519 g^2 DW g^{-1} P (usa_chi) and 59577 g^2 DW g^{-1} P (bol_144) (Supp. Table 3). This parameter averaged 25884 g^2 DW g^{-1} P under Control and 32927 g^2 DW g^{-1} P under NoP conditions. Despite that, significant differences between treatments for PPUE were only found for two accessions; mu_esp significantly decreased its PPUE by 41.31%, whereas sp_cwr increased it by 97.53% when cultivated under NoP conditions (Table 4). Regarding PER, stress treatment produced a generalized increase, averaging 87.64%, of this parameter's values. We observed values ranged between 330 g DW g^{-1} P (usa_chi) and 595 g DW g^{-1} P (bol_144), averaging 424 g DW g^{-1} P when under stress conditions. Under Control conditions values ranged between 505 g DW g^{-1} P (usa_chi) and 1060 g DW g^{-1} P (bol_144) under NoP conditions, averaging 776 g DW g^{-1} P. Only two accessions were not affected by treatment regarding PER, bol_144 and sp_bola (Table 4). Detailed information about accessions performance can be found within Supp. Table 3.

In addition, an interesting amount of variability was observed for PUpE and PUtE parameters (Figure 3). PUpE refers to the increase in P when P is present in the treatment applied, whereas PUtE measures the ability of a genotype to increase its biomass in higher proportion to an increasing amount of internal P. Regarding PUpE, values ranged between 50.51 mg P (bol_144) and 144.43 mg P (mu_esp), and averaged 96.56 mg P. Accessions mex_scm (128.77 mg P), mu_esp (144.43 mg P), sp_cat (130.06 mg P), sp_piq (124.53 mg P), and usa_chi (108.70 mg P) showed the highest values in our experiment (Figure 3). PUtE's values ranged from 29.77 g DW g^{-1} P (bol_144) to 404.95 g DW g^{-1} P (mex_pas) and averaged 186.55 g DW g^{-1} P. Accessions mex_pas (404.95 g DW g^{-1} P), usa_chi (316.77 g DW g^{-1} P) and sp_bola (273.16 g DW g^{-1} P) presented the most interesting results (Figure 3). This means that these accessions increase their yields when the amount of available P also increases and therefore are very responsive to high input environments.

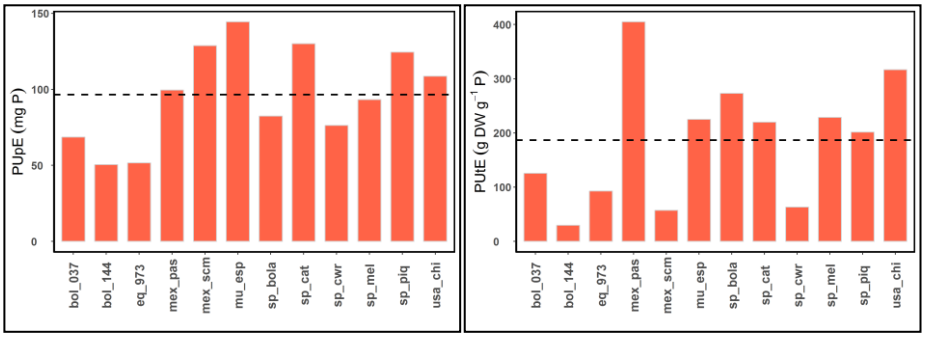

Figure 3 - Average PUpE (P Uptake Efficiency) and PUtE (P Utilisation Efficiency) values for the twelve accessions studied in Trial 1. Black dashed line represents average value for the whole collection for both PUpE (96.56 mg P) and PUtE (186.55 g DW g^{-1} P) parameters.

Treatment effect on root and shoot morphometrics for Trial 1

P is a major factor controlling root structure and architecture (Niu et al., 2013). Hence, in order to understand the possible effects on plant morphology and root structure and architecture resulting from lack of P, we compared several root and shoot traits and parameters between treatments.

Treatment and accession showed in the multifactorial ANOVA (Table 4) a significant influence over all studied traits but RHW%, where only existed significant differences among the accessions. In addition, for lateral root specific length (LRSL) parameter there was a significant accession per treatment interaction (Table 4).

Table 4 - Multi-factor ANOVA mean square values of accession (A) and treatment (T) effects, their interaction, and error (E) for and for morphometric traits RW (g), LRW (g), RHW(g), SW (g), BW (g), RSR, LRL (m), LRAD (mm), RHW% (%), PLFR (%), and LRSL (m/g) for Trial 1.

Effect	RW [a]	LRW	RHW	SW	BW	RSR	LRL	LRAD	RHW (%)	PLFR	LRSL
A	21.82***	9.86***	3.41***	487***	976**	0.021***	144***	0.042***	0.048***	0.013***	74.13***
T	62.61***	26.99***	6.92*	4237***	14799***	0.065***	176*	0.065*	0.009 ns	0.053***	456***
A x T	1.94 ns	1.23 ns	0.90 ns	149 ns	632 ns	0.006 ns	43.31 ns	0.013 ns	0.016 ns	0.002 ns	29.17*
E	3.94	1.40	1.18	129	392	0.004	41.79	0.009	0.011	0.003	7.64

[a] ***, **, * and ns indicate significant for $P<0.001$, $P<0.01$, $P<0.05$, and non-significant, respectively, obtained after analysis of variance (ANOVA) for individual accession values.

Thus, roots (RW) showed a generalised loss of dry weight (-24.52%) when genotypes were cultivated under NoP conditions, compared to Control replicas (Table 5). The significantly affected accessions were mex_pas (-36.24%), mu_esp (-46.63%), sp_cwr (-19.90%), and usa_chi (-53.35%) (Table 5). Interestingly, accession mex_scm, presented similar RW under both treatments, while presenting the biggest root system within our collection under NoP conditions (Supp. Table 3). For mex_pas, significant loss of weight affected mostly its LRW, whereas for mu_esp it was RHW that was significantly affected. In the case of usa_chi, the RW loss was significantly felt in both root components LRW and RHW (Table 5). And finally, despite RW had no significant changes, sp_bola's LRW was significantly affected by treatment. Regarding SW, significant differences were found between stress and Control treatments, and accessions presented a generalised loss of SW under NoP, averaging 36.04% (Table 4). These results were statistically significant for accessions mex_pas (-59.65%), mu_esp (-56.71%), sp_piq (-39.38%), and usa_chi (-61.36%) (Table 5). Mex_scm presented the highest SW of the whole collection under both treatments, and just like for RW, this accession was able to maintain a similar shoot biomass despite having less resources under NoP treatment (Supp. Table 3). Furthermore, BW was significantly affected by NoP treatment and was reduced by 33.56%, on average. Accessions mex_pas (-54.20%), mu_esp (-55.84%), sp_piq (-38.84%), and usa_chi (-59.42%) presented significant differences between treatments for BW. Finally, RSR was positively affected under NoP treatment. This parameter increased by 20.94%, on average, for our collection, although, only usa_chi was significantly affected (+22.73%) under NoP treatment (Table 5). Apparently, achieved by reducing shoot's weight instead of increasing root's mass (Supp. Table 3).

Regarding root trait LRL, NoP treatment yielded roots, on average, 16.65% longer than the Control treatment. Under stress treatment, sp_cwr responded with a significant increase of LRL (+65.62%) (Table 5). Furthermore, LRAD was found slightly lower (-6.29%) under NoP treatment. Accession bol_144 (-21.81%) significantly drop from 0.75 mm, under Control conditions, to 0.58 mm under NoP conditions, while no significant differences were detected for the rest of accessions (Table 5 and Supp. Table 3). Regarding RHW%, root hairs represented 7.46% more of RW under NoP compared to Control replicas, although, no significant differences were found between treatments for any accessions under our conditions. Likewise, PLFR increased, on average, 4.88% under NoP conditions. Accessions bol_144 (+9.56%), mu_esp (+7.36%), sp_bola (+8.55%), and sp_cat (+6.58%) were significantly affected by the treatment (Table 5). Finally, LRSL increased by 67.08%, on average, under NoP treatment. Significant differences between treatments were observed for mex_pas (+70.84%), mu_esp (+115.25%), sp_bola (+57.72%), sp_cwr (+91.05%) and usa_chi (+142.63%) (Table 5). Detailed information about accessions performance can be found within Supp. Table 3.

Table 5 - Accession behavior given by differences (%) when passing from Control to NoP conditions for Trial 1. P concentration traits $[P]_{tissue}$ (g P/100g DW), P content traits RootP (g P), ShootP (g P), FruitP (g P) and PTP (mg P), efficiency parameters PPUE (g^2 DW g^{-1} P) and PER (g DW g^{-1} P), and morphometric traits RW (g), LRW (g), RHW (g), SW (g), BW (g), RSR, LRL (m), LRAD (mm), RHW% (%), PLFR (%), and LRSL (m/g) were considered.

Accession	$[P]_{Root}$	$[P]_{Shoot}$	$[P]_{Fruit}$	RootP	ShootP	FruitP	PTP	PPUE	PER	RW	LRW	RHW	SW	BW	RSR	LRL	RAD	RHW%	PLFR	LRSL
bol_037	-91.62 *	-32.10	-32.56	-93.37	-37.32	-25.00	-54.72	95.23	106.37	-12.91	-8.50	-18.68	-22.16	-21.67	25.41	33.30	-8.59	-6.42	5.21	38.14
bol_144	-74.12	-34.29	-27.41	-86.11	-55.45	98.52	-51.66	90.59	78.15	-26.94	-36.58	-1.37	-8.13	-4.08	-16.22	15.14	-21.81	51.06	9.56	112.94
eq_973	-87.67	-34.32	-35.19	-87.60	-41.15	55.56	-53.92	112.72	115.10	-21.00	-30.62	-3.38	-16.21	-14.17	6.67	49.55	-16.80	39.34	6.02	78.20
mex_pas	-78.01	1.51	-19.10	-86.13	-52.54	-59.45	-63.36	-27.82	36.65	-36.24	-42.38	-26.69	-59.65	-54.20	27.89	-3.07	-9.83	12.06	6.63	70.84
mex_scm	-84.98	-44.35	-37.94	-85.62	-62.45	-24.63	-57.57	50.86	96.13	0.65	9.15	-8.95	-18.18	-12.62	21.37	8.86	7.98	-11.28	1.63	16.26
mu_esp	-77.38	-32.15	-33.93	-88.61	-77.91	-72.38	-76.17	-41.31	51.20	-46.63	-29.39	-52.87	-56.71	-55.84	9.88	26.88	-8.57	-12.76	7.36	115.25
sp_bola	-77.51	-4.11	-17.47	-75.94	-41.77	-53.31	-55.46	-0.75	38.47	-19.85	-30.22	-5.64	-43.10	-40.06	27.73	2.12	-10.84	2.89	8.55	57.72
sp_cat	-83.53	-24.27	-45.63	-83.02	-56.11	-78.90	-73.96	27.80	108.33	-2.79	-23.62	31.54	-33.26	-41.92	50.00	3.73	-13.32	25.62	6.58	20.20
sp_cwr	-90.53	-45.03	-47.21	-92.86	-55.30	-46.11	-61.87	97.53	132.69	-19.90	-14.68	-26.20	-16.17	-11.06	-6.58	65.62	-7.13	-6.42	4.17	91.05
sp_mel	-83.67	-35.14	-49.43	-87.27	-66.67	-74.04	-77.17	24.96	137.24	-30.17	-35.41	-21.27	-58.18	-48.90	63.81	-10.47	-3.90	11.05	3.12	54.84
sp_piq	-75.52	-45.26	-44.11	-78.51	-59.93	-70.57	-67.78	24.31	98.40	-25.14	-17.60	-32.96	-39.38	-38.84	18.59	-19.76	4.76	-14.57	0.98	6.84
usa_chi	-76.56	-22.25	-32.20	-88.89	-66.88	-61.56	-65.95	-18.94	52.95	-53.35	-53.03	-53.63	-61.36	-59.42	22.73	27.85	12.59	-1.07	-1.29	142.63
Global mean	-81.76	-29.31	-35.18	-86.16	-56.12	-34.32	-63.30	36.26	87.64	-24.52	-26.07	-18.34	-36.04	-33.56	20.94	16.65	-6.29	7.46	4.88	67.08

* Values highlighted in yellow indicate significant differences between treatments for that accessions and trait.

Principal components analysis for Trial 1

PCA was pursued in order to determine possible correlations between the response (increase or decrease) of the different measured traits to the different inputs of P and to explicitly demonstrate how accessions differed in terms of response to the stress treatment. The first two principal components (PC) explained 59.79% of total variability (Figure 4). PC1 (36.96%) was positively correlated with traits BW, PPUE, PUpE and FruitP while being negatively correlated with LRAD and RootP traits. In addition, PC2 (22.83%) was positively correlated to LRW, LRAD, RW and PUtE whereas $[P]_{Shoot}$, $[P]_{Fruit}$ and ShootP were negatively correlated with PC2 (Figure 4). Thus, accessions were distributed along PCs axis based on the trait's correlation with PCs. As a result, accessions mu_esp, usa_chi, mex_pas, and sp_piq showed positive correlation with PC1, on the positive side of the graph, and correlated with BW, PPUE, PUpE and FruitP (Figure 4). On the opposite side of PC1, non-*annuum* accessions (bol_144, eq_973 and bol_037) and sp_cwr showed a negative correlation with PC1 and, therefore, correlated with LRAD, and RootP (Figure 4). Of those accessions, bol_037, and sp_cwr showed a similar behaviour. Furthermore, mex_pas and sp_bola were positively correlated to PC2 and, consequently, placed at the top right quadrant of the graph, correlated to LRW, LRAD, RW and PUtE. Finally, mex_scm was negatively correlated to PC2 and placed at the bottom the plot, correlated to ShootP, $[P]_{Shoot}$, $[P]_{Fruit}$ and ShootP traits (Figure 4).

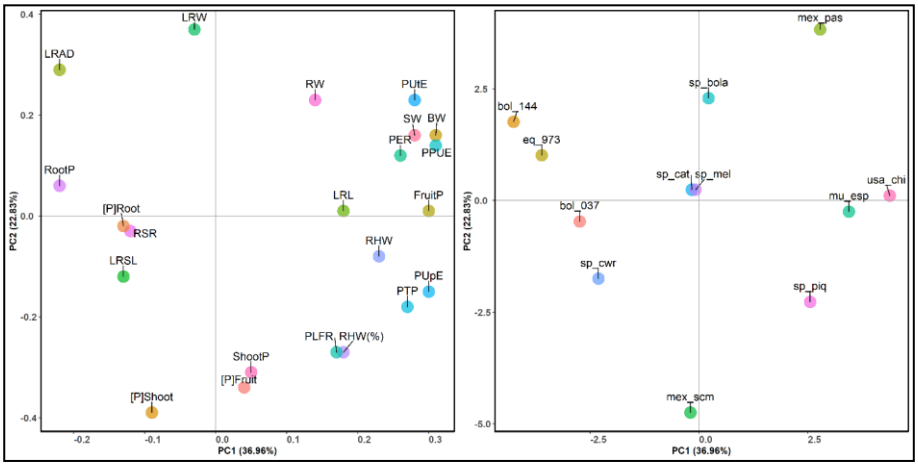

Figure 4 - PCA for the first two components based on trait differences between treatments for Trial 1. On the left, correlation between traits and the first two principal components. On the right, distribution of accessions based on studied traits. P tissue concentration traits $[P]_{Tissue}$, P tissue total content traits RootP, ShootP, FruitP, P total plant content (PTP) trait, efficiency parameters PPUE (physiological P use efficiency), PER (P efficiency ratio), PUpE (P uptake efficiency) and PUtE (P utilization efficiency), and morphometric traits RW (root dry weight), LRW (lateral root dry weight), RHW (root hairs dry weight), SW (shoot dry weight), BW (total biomass dry weight), RSR (root to shoot ratio), LRL (lateral root length), LRAD (lateral root average diameter), RHW% (root hairs dry weight %), PLFR (proportion of length dedicated to fine roots), and LRSL (lateral root specific length) were considered.

Treatment effect on accumulated P and PUE parameters for Trial 2

In Trial 2, five accessions from Trial 1 were essayed against 13 new *C. annuum* accessions under the same Control and NoP treatments and mesh greenhouse of Trial 1. Hence, accessions mu_esp, mex_pas, sp_bola, sp_piq, and mex_scm were re-trialled and used as comparison standard to evaluate the performance of new accessions.

The multifactorial ANOVA showed that treatment effect was the most important, although there was a significant accession effect for all P accumulation and PUE traits and parameters (Table 6). Interestingly, PPUE was more affected by the accession effect, in this Trial. Finally, interaction between accession and treatment effects was highly significant for PTP and PPUE ($P<0.001$), and significant ($P<0.05$) for PER (Table 6).

Table 6 - Multi-factor ANOVA's mean square values of accession (A) and treatment (T) effects, their interaction and error (E) for P concentration trait $[P]_{Shoot}$, P content trait PTP (mg P), for efficiency parameters PPUE (g^2 DW g^{-1} P) and PER (g DW g^{-1} P), and for morphometric traits RW (g), LRW (g), RHW (g), SW (g), BW (g), RSR, RTL (m), TAD (mm), RHW%, PFR (%), and RSL (m/g) for Trial 2.

Effect	$[P]_{Shoot}$[a]	PTP	PPUE	PER	RW	LRW	RHW	SW	BW	RSR	RTL	TAD	RHW%	PFR	RSL
A	0.075***	95685***	1.15E9***	10766***	7.66***	1.51***	4.59***	1752***	1939***	0.003***	14.47***	0.019***	0.074***	0.010***	2.53***
T	2.600***	4607020***	5.06E8***	270121***	274***	12.23***	170.66***	59110***	66680***	0.000 ns	4.53 ns	0.013 ns	0.469***	0.030***	91.68***
A x T	0.017 ns	94464***	4.41E7***	2158*	4.42**	0.593***	2.68*	1669***	1815***	0.001 ns	2.78 ns	0.005 ns	0.008 ns	0.002 ns	1.20 ns
E	0.012	21148	1.58E+07	1189	1.91	0.158	1.32	446	484	0.001	3.77	0.004	0.011	0.002	0.72

[a] ***, **, * and ns indicate significant for *P*<0.001, *P*<0.01, *P*<0.05, and non-significant, respectively, obtained after analysis of variance (ANOVA) for individual accession values.

$[P]_{Shoot}$ was significantly affected in 16 of 18 assessed accessions and values under NoP were 31.50% lower than under Control conditions (Table 7). Considering PTP, accessions showed a significant loss, averaging 66.17%, when cultivated under NoP, compared to Control replicas (Table 7). Accessions mex_sandia (-87.88%), sp_piq (-86.93%) and mex_scm (-84.16%) presented the biggest difference between Control and NoP treatments, whereas sp_lam and sp_lobo showed no differences between treatments (Figure 5 and Table 7).

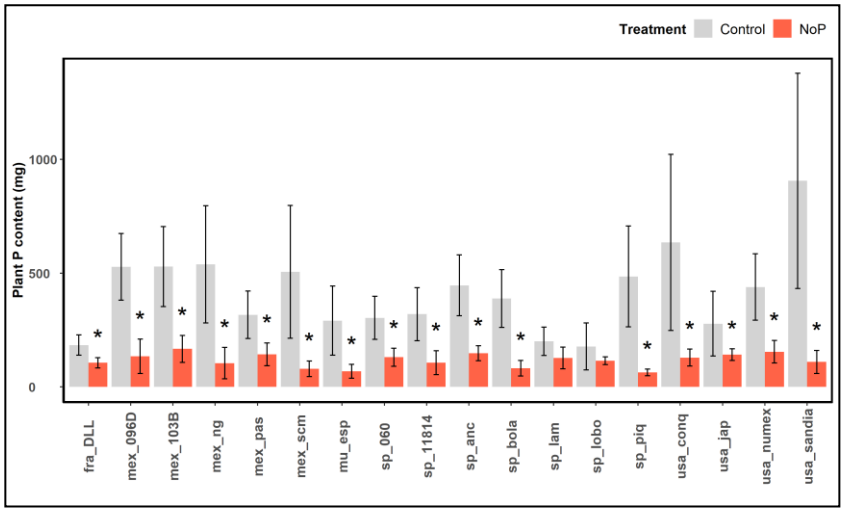

Figure 5 - PTP (mg) mean values presented by accessions for both Control and NoP treatments for Trial 2. Asterisks indicate significant differences (P<0.05) between treatments for a given accession, whereas absence of asterisks indicates no significant differences between treatments were found. In addition, black bars indicate standard deviation for a particular accession and treatment.

Regarding PPUE, values averaged 9337 g^2 DW g^{-1} P and 6249 g^2 DW g^{-1} P for Control and NoP conditions, respectively, being on average 24.98% lower under NoP conditions (Table 7 and Supp. Table 4). Accessions mex_scm (-72.75%), usa_sandia (-64.94%) sp_piq (-50.75%), mex_103B (-42.41%), and mex_pas (-39.68%) were significantly affected by the stress treatment regarding PPUE parameter. Highest values were presented by usa_sandia (17345 g^2 DW g^{-1} P) and fra_DLL (13566 g^2 DW g^{-1} P) for Control and NoP conditions, respectively. PER showed higher values under NoP, comparing to Control conditions, increasing 49.26%, on average (Table 7). This parameter averaged 150 g DW g^{-1} P and 222 g DW g^{-1} P for Control and NoP conditions, respectively. All accessions, except mex_pas and sp_lobo, were significantly affected by

treatment. Accessions sp_piq (+91.29%) and mex_096D (+87.88%) presented the biggest increment for this parameter (Table 7). Highest observed PER values were presented by fra_DLL for both treatments, showing 235 g DW g^{-1} P under Control treatment and 350 g DW g^{-1} P under NoP (Supp. Table 4).

Regarding PUpE, average value was 298 mg P, ranging from 63 mg P (sp_lobo) to 796 mg P (usa_sandia). Accessions usa_sandia (796 mg P) and usa_conq (506 mg P) presented the highest values for this parameter (Figure 6). Finally, average PUtE was 110 g DW g^{-1} P while the minimum observed value was 43 g DW g^{-1} P (sp_lam) and the maximum was 183 g DW g^{-1} P (mex_pas). Accessions mex_pas (183 g DW g^{-1} P) and sp_bola (147 g DW g^{-1} P) presented the best performance for this parameter (Figure 6).

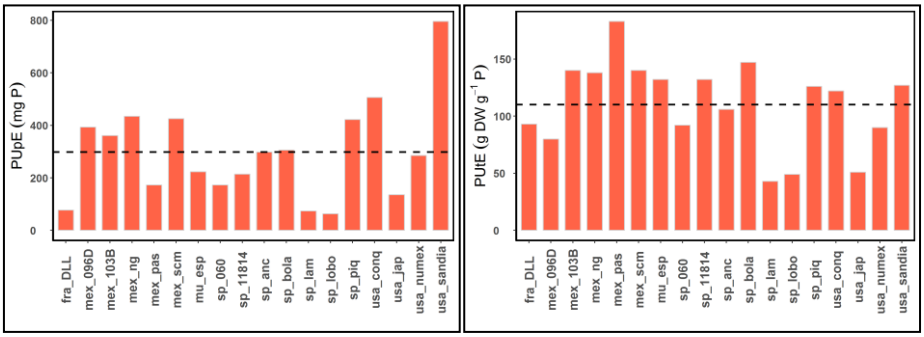

Figure 6 - Average PUpE and PUtE values for the twelve accessions studied in Trial 2. Black dashed line represents average value for the whole collection for both PUpE (298 mg P) and PUtE (110 g DW g^{-1} P) parameters.

Treatment effect on root and shoot morphometrics for Trial 2

Multi-factorial ANOVA detected significant differences for Trial 2 (Table 6). In this case, treatment effect was significant for almost all traits except for RSR, RTL and TAD. In addition, accession effect was significant for all traits, and a significant treatment x accession interaction was detected for RW, LRW, RHW, SW and BW traits (Table 6).

Regarding traits performance, roots showed a generalised loss of dry weight (-52.96%) when genotypes were cultivated under NoP conditions, compared to the Control replicas. Most accessions were significantly affected, and the most affected genotypes were sp_piq (-72.33%) and mex_scm (-71.09%). Accessions fra_DLL, sp_lam, sp_lobo and usa_jap were not significantly affected (Table 7). Naturally, the loss of root weight is linked to a reduction of LRW or RHW, or even in both components. We observed different behaviours among accessions, for instance, sp_anc RW loss was due to a

reduction of its LRW weight, for mex_96D, mu_esp, sp_060, sp_11814, sp_bola, usa_conq, usa_jap and usa_numex RW loss was due to a significant loss of RHW, and finally mex_103B, mex_ng, mex_pas, mex_scm, sp_piq, and usa_sandia both root components were significantly affected by the treatment and reduced their total mass (Table 7). Regarding SW, accessions presented a significant loss of SW under NoP, averaging 50.18%. These results were statistically significant for the same accessions mentioned for RW and the most affected were usa_sandia (-80.15%) and mex_scm (-79.40%). Thus, BW was also negatively affected by NoP treatment and was reduced, on average, in 50.11%. Accessions fra_DLL, sp_lam, sp_lobo, and usa_jap were not affected by the treatment, whereas usa_sandia (-79.31%) and mex_scm (-78.97%) presented the biggest drop in BW (Table 7). Finally, parameter RSR was, on average, 6.80% higher under NoP treatment than under Control conditions. However, this parameter was not significantly affected for most accessions and, surprisingly, for the ones it did, those accessions showed a decrease. Thus, sp_060 (-24.24%), sp_lam (-30.56%) and usa_jap (-45.95%) were significantly affected by the low P conditions (Table 7).

Furthermore, RTL was 13.42% longer under NoP than under Control, on an average. Accession usa_numex (-39.33%) was the only genotype significantly affected by treatment effect for this trait (Table 7). TAD was also not significantly affected by treatment for most accessions, although it showed a slight decrease under stress conditions (-1.91%). Accessions mex_103B (-7.53%), mex_ng (-13.30%) and mu_esp (-8.37%) significantly decreased their TAD under NoP conditions. Likewise, RHW% was 14.03% lower under P-stress conditions with accessions mex_096D (-19.79%), sp_11814 (-21.27%), sp_bola (-26.14%), and sp_piq (-21.96%) being the significantly affected ones (Table 7). Parameter PFR showed a slight increase under NoP (3.33%), compared to Control conditions, although only four accessions were significantly affected. Thus, accessions mex_103B (+5.91%), mex_ng (+9.79%) and mu_esp (+6.53%) and sp_lobo (+8.97) significantly increased this parameter under stress. Ultimately, RSL was, on average, 138.71% higher under NoP conditions. Most accessions were significantly affected by treatment, mu_esp (+293.57%) and sp_11814 (+282.31%) were the accessions with higher increase for root specific length (Table 7). Detailed information about accessions individual performance, such as mean values and deviations, is available in Supp. Table 4.

Table 7 - Accession behavior given by differences (%) when passing from Control to NoP conditions for Trial 2. P concentration trait $[P]_{Shoot}$, P content trait PTP (mg), efficiency parameters PPUE (g^2 DW g^{-1} P) and PER (g DW g^{-1} P), and morphometric traits RW (g), LRW (g), RHW (g), SW (g), BW (g), RSR, RTL (m), TAD (mm), RHW%, PFR (%), and RSL (m/g) were considered.

Accession	[P]Shoot	PTP	PPUE	PER	RW	LRW	RHW	SW	BW	RSR	RTL	TAD	RHW%	PFR	RSL
fra_DLL	-31.92*	-42.13	25.76	48.75	-31.04	-20.87	-34.20	-14.49	-15.61	-20.51	6.27	4.23	-6.84	-0.31	58.00
mex_096D	-44.10	-74.54	8.80	87.88	-42.62	-29.17	-50.76	-49.18	-48.86	27.27	-0.69	2.96	-19.79	-2.46	71.79
mex_103B	-25.45	-68.35	-42.41	33.91	-63.97	-57.22	-68.38	-56.97	-57.48	-4.26	-10.44	-7.53	-10.29	5.91	131.10
mex_ng	-38.75	-80.58	-53.53	66.18	-70.25	-68.60	-71.38	-69.72	-69.75	7.95	-0.06	-13.30	-7.35	9.79	229.58
mex_pas	-11.63	-54.71	-39.68	14.69	-45.43	-37.30	-52.56	-48.13	-47.97	13.16	6.22	-0.80	-11.40	1.83	81.25
mex_scm	-24.46	-84.16	-72.75	30.99	-71.09	-53.17	-79.62	-79.40	-78.97	60.71	-15.45	-0.72	-21.79	2.51	96.42
mu_esp	-28.78	-76.55	-45.93	41.15	-58.48	-36.78	-66.55	-64.64	-64.02	33.93	83.39	-8.37	-16.18	6.53	293.57
sp_060	-23.21	-57.00	-19.84	30.93	-58.36	-32.94	-64.94	-41.59	-41.30	-24.24	17.90	-1.52	-18.90	5.90	181.31
sp_11814	-27.72	-66.80	-40.61	34.34	-54.26	-9.84	-65.36	-55.36	-54.65	-6.46	110.07	-0.85	-21.27	3.37	282.31
sp_anc	-35.55	-66.88	-20.37	53.60	-47.93	-37.11	-53.30	-48.66	-48.08	2.94	-9.82	-5.51	-7.61	6.16	44.62
sp_bola	-29.23	-78.82	-59.09	41.52	-63.73	-6.21	-73.15	-70.90	-70.35	23.08	52.67	-3.10	-26.14	4.62	261.22
sp_lam	-30.84	-36.71	29.12	42.54	-35.96	-24.57	-42.23	-7.24	-10.66	-30.56	-7.73	2.45	-8.40	0.22	38.68
sp_lobo	-24.83	-35.31	16.75	31.29	-13.58	-9.56	-15.06	-14.14	-10.95	6.90	20.75	-7.69	-0.93	8.97	27.40
sp_piq	-42.48	-86.93	-50.75	91.29	-72.33	-51.80	-79.12	-75.78	-75.48	8.33	4.58	3.29	-21.96	2.35	220.45
usa_conq	-38.95	-79.74	-47.09	61.46	-59.27	-41.08	-65.12	-67.50	-66.97	13.04	3.73	-8.02	-14.25	8.23	122.83
usa_jap	-39.15	-49.02	45.04	68.30	-48.83	-23.47	-60.76	-13.55	-15.53	-45.95	9.01	-0.44	-21.29	0.00	72.51
usa_numex	-33.47	-64.88	-17.21	50.09	-49.78	-38.25	-53.04	-45.86	-46.10	-9.52	-39.33	8.76	-7.89	-3.70	21.77
usa_sandia	-36.52	-87.88	-65.94	57.77	-66.39	-59.05	-68.20	-80.15	-79.31	66.67	10.55	1.80	-10.35	0.10	262.06
Global mean	-31.50	-66.17	-24.98	49.26	-52.96	-35.39	-59.10	-50.18	-50.11	6.80	13.42	-1.91	-14.03	3.33	138.71

* Values highlighted in yellow indicate significant differences between treatments for that accessions and trait.

Principal components analysis for Trial 2

The first two PCs explained 63.84% of total variation for our collection for Trial 2. PC1 (47.48%) was positively correlated with BW, SW, PPUE, PUpE, PTP, RW, and RHW while being negatively correlated with RSR and RSL traits (Figure 7). PC2 (16.36%), on the other hand, was positively correlated with $[P]_{Shoot}$, PFR and RTL while being negatively correlated with TAD and PER (Figure 7). Based on those results, accessions usa_sandia was positively correlated to PC1 and placed at the right side of the graph. On the opposite side were located sp_lam, fra_DLL, sp_lobo and usa_jap accessions, correlated to RSR and RSL traits (Figure 7). Regarding PC2, accessions usa_numex and mex_096D were positively correlated and placed at the top of the plot, linked to $[P]_{Shoot}$, PFR and RTL. On the other end of the axis, accessions mu_esp, mex_pas and sp_11814 were located in correlation with TAD and PER (Figure 7).

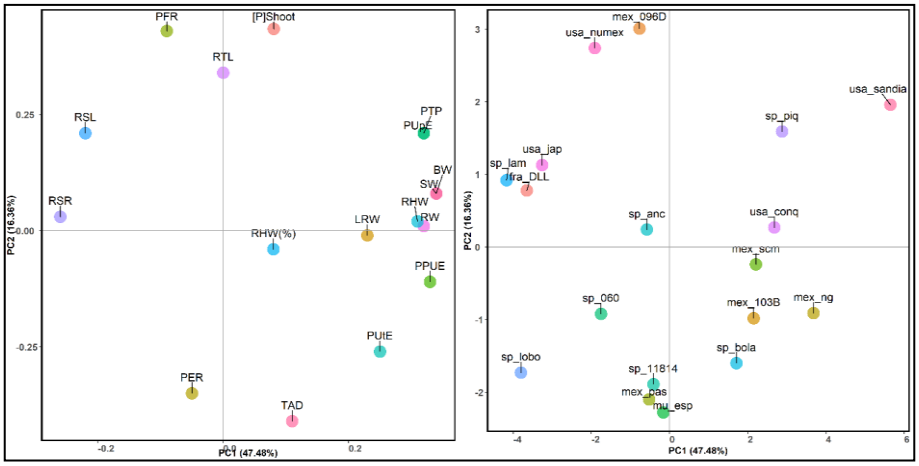

Figure 7 - PCA based on trait increments between treatments for the Trial 2 experiment. On the left, correlation between traits and the first two principal components. On the right, distribution of accessions based on the studied traits. P tissue concentration traits $[P]_{Shoot}$, P total plant content (PTP) trait, efficiency parameters PPUE (physiological P use efficiency), PER (P efficiency ratio), PUpE (P uptake efficiency) and PUtE (P utilization efficiency), and morphometric traits RW (root dry weight), LRW (lateral root dry weight), RHW (root hairs dry weight), SW (shoot dry weight), BW (total biomass dry weight), RSR (root to shoot ratio), RTL (root total length), TAD (root total average diameter), RHW% (root hairs dry weight %), PFR (proportion of length dedicated to fine roots), and RSL (root specific length) were considered.

Discussion

Treatment effect on P and other minerals concentrations

Comparison of P (root, shoot and fruit in Trial 1 and just shoot in Trial 2) concentrations in plant tissues provided relevant information to affirm that NoP treatment was effective and significantly different from Control treatment (Supp. Table 2). In addition, after P low input treatment, our results indicate a significant decrease of P concentration in all plant tissues, an expected behaviour already reported by other authors for other crops (Fernandez and Rubio, 2015). Furthermore, there is evidence to suggest that pepper plant organs require P in different amounts, and the minimum levels are drastically different between tissues. Regarding that, roots presented the highest drop for P concentration between treatments, indicating that it is able to mobilize P in order to benefit above ground biomass (Supp. Table 2). Other reports observed that root porosity and aerenchyma proportion increased under P-starvation conditions, resulting in a reduction of both the metabolic expenses and P requirements of the root system while maintaining foraging capacity (Fan et al., 2003; Fernandez and Rubio, 2015). Adding to that, there were differences among genotypes on P allocation, which opens the door to breed materials with less P in the fruits less need for fertilization. Some authors believe that we consume more P than the required for a healthy diet, and often under the form of phytate, which is not fully assimilable by our digestive system (Rose et al., 2010).

Finally, despite the existent significant differences between treatments for other macrominerals and tissue combinations, values observed for our experiment are within the normal range, and therefore no deficiency or excess was detected apart from P (Jones, 2012; Russo, 2012) (Supp. Table 2). Likewise, homeostatic processes by which plants uptake, transport and store nutrients are not independent, and therefore, the absence or excess of some elements can affect how the rest are processed (Bouain et al., 2014; Jones, 2012).

Treatment effect on PTP and P efficiency parameters

Stress treatment yielded significantly lower P accumulation, compared to Control, for both trials, as indicated by PTP (Tables 3 and 5). Both trials showed an average reduction of around 65% for PTP, supported by the common accessions' behaviour, which showed a similar response between trials (Tables 3 and 5). This is consistent with previous works, which reported a significant drop of P levels under P-stress conditions (Ao et al., 2010; Fernandez and Rubio, 2015; Fita et al., 2011).

Furthermore, the use of parameters to describe plant's mineral uptake and use efficiency is a widely adopted practice in this scientific field (Fita et al., 2011; Hammond et al., 2009). Hence, PPUE provides information on how productive a genotype may be, based on its tissues P concentration under a specific treatment; thus, high values indicate higher efficiency transforming absorbed P into biomass. Under our conditions, accessions mex_pas (Control) and bol_144 (NoP) presented the highest PPUE for Trial 1, whereas in Trial 2, usa_sandia and fra_DLL presented the highest values for Control and NoP, respectively (Supp. Tables 3 and 4). These results indicate a differential response, making these accessions interesting candidates for different fertilizers input conditions (e.g. high and low). Interestingly, trials provided contrasting results for PPUE. For Trial 1 accessions increased around 36% whereas in Trial 2 PPUE mean value drop almost 25%, where mex_scm, sp_bola and sp_piq drastically changed their response (Tables 3 and 5). Bear in mind that although it's the same parameter, it is calculated in a different way. For Trial 1 it is calculated as a mean for all plant tissues whereas for Trial 2 we extrapolated from shoot tissue, which may have caused behaviour distortion. PER parameter, on the other hand, relates the amount of yielded biomass with the amount of accumulated P in the plant, so high values indicate a higher ability to generate biomass with less P. Thus, bol_144 (Trial 1) and fra_DLL (Trial 2) are extremely efficient genotypes, especially under low input conditions (Supp. Tables 3 and 4). These results indicate an interesting ability to use every unit of absorbed P and convert it into Biomass and the aptitude of these genotypes in low input systems.

Regarding PUpE, accessions mex_scm and sp_piq showed an above average performance in both trails, although in the Trial 2 both usa_conq and usa_sandia showed higher values. This indicates that these accessions respond well to fertilization and are able to uptake high amounts of P when it is present. In terms of PUtE, accessions mex_pas and sp_bola showed the highest values in both trials, indicating that are able to use more efficiently the P they absorb into biomass generation than the rest of accessions. Furthermore, genetic variation regarding P acquisition and use efficiency has been widely reported in many other crops (Akhtar et al., 2008; Fernandez and Rubio, 2015; Fita et al., 2011; Hammond et al., 2009; Hu et al., 2010). However, to our knowledge, this is the first work that provides such information for pepper germplasm. Herein we report a wide range of variability regarding P efficiency parameters as well

as several combinations among them, offering numerous possibilities for breeding for improved PUE. Several authors have reported independence between uptake and use efficiency, which enables the improvement of both as well as selecting materials with different purposes (e.g. high and low input environments) (Fita et al., 2011; Hammond et al., 2009; Hu et al., 2010; Lynch, 2007). Our results seem to point towards that idea, since both parameters located separately in both trials PCA (Figures 4 and 7).

Treatment effect on root and plant traits

Our results indicate that apart from biomass loss caused by P deficiency, there are important modifications, particularly at root level, that help the plant to cope with P stress. This was also observed in previous works with other crops for P-starvation conditions (Niu et al., 2013). Furthermore, many genotypes promote root growth instead of aerial in order to enhance its foraging capability (Fernandez and Rubio, 2015; Li et al., 2009). In our work we observed a significant loss of RW compared to Control irrigated plants, however, this behaviour could mean that genotypes favoured the formation of thinner roots with increased porosity and aerenchyma in their structure (Fan et al., 2003). This decrease of root diameter is a typical response under low input P conditions and consists in the stimulation of root hairs instead of primary roots (Bates and Lynch, 2001; Fernandez and Rubio, 2015; Hammond et al., 2009) while also halting secondary growth of the root (Strock et al., 2018). Other works have also reported loss of root weight under P-stress conditions (Fernandez and Rubio, 2015; Fita et al., 2011).

The labour inherent to root data collecting is hard and tedious. Despite that, roots were carefully separated from the soil, in order to keep damage to a minimum; it is possible that a significant amount of root hairs and thin lateral roots ended up being ripen from the root system, resulting in lack of significance of treatment for RHW%. Despite that, our results are in agreement with other reports on increase of LRL, PLFR/PFR and RSL as well as the decrease of root's diameter under P-stress treatment (Bates and Lynch, 2001; Fernandez and Rubio, 2015; Hammond et al., 2009; López-Bucio et al., 2003). Morphological adaptations to low P concentrations in the soil aim at enhancing P acquisition without significantly increase of metabolic costs. In that way, increment of lateral roots length, root hairs and root biomass have been reported to enable exploitation of a bigger soil volume as well as to enhance P uptake.

Trait correlations provided by PCA

Our results indicate a positive correlation among response of morphological and P accumulation traits with P-uptake and P-use efficiency parameters (Figures 4 and 7).

For example, in both trials, PUpE and PUtE parameters were positively correlated with increases in biomass accumulation and incremental P content in plant tissues. Likewise, positive correlation was found between the increase of root hairs parameters and PUpE and PUtE parameters, reinforcing the idea that root hairs have higher ability to absorb P and because smaller roots consume less P that increases the resources that can be allocated to biomass yield (Figure 4 and 7) (Miguel et al., 2015; Niu et al., 2013). Contrastingly, increases of RSR and RSL/LRSL were negatively correlated to increases of above ground biomass and PUE parameters, and, in Trial 1, even correlated to increases of RootP and LRAD (Figure 4). This does not necessarily imply that RSR or RSL are not important parameters, regarding pepper adaptation to different P levels. Instead, it implies that genotypes showing high differences between treatments for these parameters were not efficient, probably because truly efficient genotypes had already good values under Control conditions. PUpE and PUtE seem to be controlled independently and here it is demonstrated by the positioning of both parameters in opposite quadrants of PC2, opening the possibility to breed towards different goals (Fita et al., 2011; Hammond et al., 2009; Hu et al., 2010; Lynch, 2007).

PCA's projection showed a widely differentiated behaviour among accessions where, for example, some responded to the treatment by increasing RSR, others by reducing LRAD, among other. On that matter, the availability of diversity is of paramount importance for crop breeding, enabling the combination of several favourable traits or behaviours in a single genotype, which in return can be a more effective solution that to have those traits separate. For example, Miguel et al. (2015), in common bean, demonstrated that combining shallow basal roots and long root hairs yielded a larger effect regarding P acquisition than their additive effects separately.

Conclusions

Herein we characterized a diverse collection of peppers for its behaviour under P starvation conditions. We report a considerable amount of diversity for response to the stress treatment for several root traits related to enhanced P acquisition and to use efficiency. An exhaustive characterization of pepper germplasm for improved root architecture and enhanced P use efficiency is of paramount importance in order to understand the mechanisms controlling the response and consequently be able to use them to develop improved varieties. However, exhaustive characterization of root traits is a laborious and tedious task and a major challenge, where small mistakes can have a major impact on conclusions. To our knowledge, this is the first time that the response of pepper to low P input conditions is studied.

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Supplementary data

Supp. Table 1 - Ion concentrations for irrigation water, Control and NoP solutions used in both Trial 1 and Trial 2.

	Ions	Irrigation Water	Control solution	NoP solution
Anions (mMol/L)	NO_3^-	0.11	15.40	15.40
	$H_2PO_4^-$	0.00	1.50	0.00
	SO^{2-}_4	2.45	0.00	0.00
	HCO^-_3	3.10	0.00	0.00
	Cl^-	1.61	0.00	0.00
Cations (mMol/L)	NH^+_4	0.00	1.50	1.50
	K^+	0.40	1.50	6.10
	Ca^{2+}	3.15	1.60	1.60
	Mg^{2+}	1.51	1.75	1.00
	Na^+	0.00	0.00	0.00

Supp. Table 2 - Effect of Control and NoP treatments on P, K, Ca, Mg, Na, and S (g/100g DW) concentrations for root, shoot, and fruit tissues for Trial 1. Overall mean values, standard deviation, and P-value for each plant tissue and treatment are provided.

Tissue	Treatment	P (g/100g DW)	K (g/100g DW)	Ca (g/100g DW)	Mg (g/100g DW)	Na (g/100g DW)	S (g/100g DW)
Root	Control	0.56 ± 0.22	0.39 ± 0.45	2.62 ± 0.62	0.30 ± 0.14	0.13 ± 0.10	0.35 ± 0.10
	NoP	0.10 ± 0.05	0.39 ± 0.54	1.72 ± 0.51	0.23 ± 0.07	0.09 ± 0.04	0.27 ± 0.05
	P-value*	0.000	0.9867	0.000	0.001	0.021	0.000
Shoot	Control	0.18 ± 0.05	3.87 ± 0.56	2.07 ± 0.54	1.02 ± 0.25	0.06 ± 0.08	0.43 ± 0.08
	NoP	0.12 ± 0.03	2.47 ± 0.93	1.95 ± 0.57	0.89 ± 0.28	0.06 ± 0.07	0.40 ± 0.06
	P-value	0.000	0.000	0.3186	0.0202	0.8782	0.0878
Fruit	Control	0.26 ± 0.06	1.15 ± 0.17	0.15 ± 0.06	0.17 ± 0.03	0.08 ± 0.09	0.20 ± 0.03
	NoP	0.17 ± 0.04	2.12 ± 0.39	0.14 ± 0.05	0.14 ± 0.03	0.06 ± 0.06	0.18 ± 0.04
	P-value	0.000	0.000	0.3809	0.0001	0.1786	0.0006

* P-value < 0.05 indicates a significant difference according to F-statistics and a 95% confidence interval.

Supp. Table 3 (a) - Trial 1 mean values and standard deviation for P tissue concentration traits [P]Tissue (g/100g DW) , P content traits RootP (g), ShootP (g), FruitP (g) and PTP (mg), efficiency parameters PPUE (g^2 DW g^{-1} P) and PER (g DW g^{-1} P), and morphometric traits RW (g), SW (g), BW (g), RSR, LRL (m), RAD (mm), RHW%, PLFR (%), and LRSL (m/g) were considered.

Abbreviation	[P]Root (g/100g DW)					[P]Shoot (g/100g DW)					[P]Fruit (g/100g DW)				
	Control	[1]	*NoP*		*Treatment P-value* [2]	*Control*		*NoP*		*Treatment P-value*	*Control*		*NoP*		*Treatment P-value*
bol_037	0.87 ± 0.32	b	0.07 ± 0.05	a	0.003	0.19 ± 0.06	a-c	0.13 ± 0.02	a	0.092	0.18 ± 0.02	a	0.12 ± 0.02	a	0.002
bol_144	0.39 ± 0.10	a	0.10 ± 0.02	a	0.004	0.18 ± 0.03	a-c	0.12 ± 0.01	a	0.063	0.24 ± 0.02	a-c	0.17 ± 0.07	ab	0.136
eq_973	0.49 ± 0.19	ab	0.06 ± 0.01	a	0.004	0.20 ± 0.04	a-c	0.13 ± 0.07	a	0.115	0.26 ± 0.03	a-d	0.17 ± 0.04	ab	0.043
mex_pas	0.48 ± 0.18	ab	0.11 ± 0.06	a	0.018	0.12 ± 0.02	a	0.12 ± 0.03	a	0.942	0.23 ± 0.02	ab	0.18 ± 0.02	ab	0.034
mex_scm	0.44 ± 0.17	ab	0.07 ± 0.04	a	0.006	0.24 ± 0.06	c	0.13 ± 0.03	a	0.019	0.33 ± 0.04	cd	0.20 ± 0.03	ab	0.002
mu_esp	0.49 ± 0.22	ab	0.11 ± 0.04	a	0.015	0.18 ± 0.03	a-c	0.12 ± 0.03	a	0.042	0.26 ± 0.05	a-d	0.17 ± 0.04	ab	0.031
sp_bola	0.67 ± 0.23	ab	0.15 ± 0.03	a	0.004	0.13 ± 0.04	ab	0.13 ± 0.03	a	0.825	0.22 ± 0.05	ab	0.18 ± 0.05	ab	0.272
sp_cat	0.66 ± 0.15	ab	0.11 ± 0.06	a	0.001	0.15 ± 0.01	a-c	0.11 ± 0.04	a	0.127	0.24 ± 0.06	a-c	0.13 ± 0.04	a	0.016
sp_cwr	0.71 ± 0.15	ab	0.07 ± 0.01	a	0.000	0.17 ± 0.03	a-c	0.09 ± 0.02	a	0.011	0.29 ± 0.06	b-d	0.15 ± 0.03	ab	0.011
sp_mel	0.70 ± 0.24	ab	0.11 ± 0.03	a	0.003	0.18 ± 0.01	a-c	0.12 ± 0.04	a	0.019	0.25 ± 0.04	a-c	0.12 ± 0.03	a	0.004
sp_piq	0.41 ± 0.09	ab	0.10 ± 0.06	a	0.001	0.23 ± 0.07	bc	0.12 ± 0.03	a	0.040	0.30 ± 0.06	b-d	0.17 ± 0.02	ab	0.006
usa_chi	0.50 ± 0.09	ab	0.12 ± 0.05	a	0.000	0.20 ± 0.04	a-c	0.16 ± 0.02	a	0.104	0.34 ± 0.02	d	0.23 ± 0.02	b	0.000
Global mean	0.56 ± 0.22		0.10 ± 0.05			0.18 ± 0.05		0.12 ± 0.03			0.26 ± 0.06		0.17 ± 0.04		

[1] Different letters within columns indicate significant differences among accessions for a given treatment and trait according to Student-Newman-Keuls post-hoc test for $P < 0.05$.

[2] Treatment P-value indicates whether or not treatments are significantly different for a given accession and trait (within lines). The coloured values (red) indicate significant differences between treatments.

Supp. Table 3 (b) - Trial 1 mean values and standard deviation for P tissue concentration traits [P]Tissue (g/100g DW) , P content traits RootP (g), ShootP (g), FruitP (g) and PTP (mg), efficiency parameters PPUE (g^2 DW g^{-1} P) and PER (g DW g^{-1} P), and morphometric traits RW (g), SW (g), BW (g), RSR, LRL (m), RAD (mm), RHW%, PLFR (%), and LRSL (m/g) were considered.

Abbreviation	RootP (g)				ShootP (g)				FruitP (g)			
	Control [1]	*NoP*	*Treatment P-value* [2]		*Control*	*NoP*	*Treatment P-value*		*Control*	*NoP*	*Treatment P-value*	
bol_037	0.045 ± 0.020 a	0.003 ± 0.001 a	0.006		0.052 ± 0.022 a	0.033 ± 0.019 a	0.226		0.028 ± 0.013 ab	0.021 ± 0.017 a	0.546	
bol_144	0.036 ± 0.017 a	0.005 ± 0.001 a	0.025		0.067 ± 0.033 a	0.030 ± 0.008 a	0.235		0.011 ± 0.009 a	0.022 ± 0.019 a	0.344	
eq_973	0.030 ± 0.005 a	0.004 ± 0.002 a	0.000		0.056 ± 0.031 a	0.033 ± 0.013 a	0.213		0.009 ± 0.006 a	0.014 ± 0.018 a	0.611	
mex_pas	0.031 ± 0.006 a	0.004 ± 0.002 a	0.001		0.044 ± 0.012 a	0.021 ± 0.004 a	0.009		0.082 ± 0.037 ab	0.033 ± 0.021 a	0.062	
mex_scm	0.036 ± 0.006 a	0.005 ± 0.004 a	0.000		0.136 ± 0.051 b	0.051 ± 0.009 b	0.017		0.051 ± 0.028 ab	0.038 ± 0.037 a	0.612	
mu_esp	0.020 ± 0.010 a	0.002 ± 0.001 a	0.014		0.064 ± 0.021 a	0.014 ± 0.007 a	0.004		0.105 ± 0.069 b	0.029 ± 0.008 a	0.072	
sp_bola	0.033 ± 0.011 a	0.008 ± 0.004 a	0.005		0.040 ± 0.024 a	0.023 ± 0.007 a	0.227		0.076 ± 0.033 ab	0.035 ± 0.023 a	0.092	
sp_cat	0.040 ± 0.010 a	0.007 ± 0.004 a	0.001		0.045 ± 0.024 a	0.020 ± 0.004 a	0.082		0.091 ± 0.035 b	0.019 ± 0.012 a	0.009	
sp_cwr	0.035 ± 0.010 a	0.002 ± 0.001 a	0.001		0.033 ± 0.003 a	0.015 ± 0.003 a	0.001		0.056 ± 0.017 ab	0.030 ± 0.010 a	0.054	
sp_mel	0.041 ± 0.025 a	0.005 ± 0.002 a	0.029		0.052 ± 0.032 a	0.017 ± 0.013 a	0.088		0.028 ± 0.024 ab	0.007 ± 0.001 a	0.196	
sp_piq	0.030 ± 0.011 a	0.006 ± 0.005 a	0.008		0.070 ± 0.016 a	0.028 ± 0.008 a	0.003		0.083 ± 0.046 ab	0.024 ± 0.015 a	0.053	
usa_chi	0.020 ± 0.003 a	0.002 ± 0.001 a	0.000		0.038 ± 0.013 a	0.013 ± 0.003 a	0.009		0.106 ± 0.016 b	0.041 ± 0.005 a	0.000	
Global mean	0.033 ± 0.013	0.005 ± 0.003			0.059 ± 0.035	0.025 ± 0.014			0.061 ± 0.045	0.027 ± 0.018		

[1] Different letters within columns indicate significant differences among accessions for a given treatment and trait according to Student-Newman-Keuls post-hoc test for $P < 0.05$.

[2] Treatment P-value indicates whether or not treatments are significantly different for a given accession and trait (within lines). The coloured values (red) indicate significant differences between treatments.

Supp. Table 3 (c) - Trial 1 mean values and standard deviation for P tissue concentration traits [P]Tissue (g/100g DW) , P content traits RootP (g), ShootP (g), FruitP (g) and PTP (mg), efficiency parameters PPUE (g^2 DW g^{-1} P) and PER (g DW g^{-1} P), and morphometric traits RW (g), SW (g), BW (g), RSR, LRL (m), RAD (mm), RHW%, PLFR (%), and LRSL (m/g) were considered.

Abbreviation	Plant total P content (PTP, mg)					Physiological P use efficiency (PPUE, g^2 DW g^{-1} P)					P efficiency ratio (PER, g DW g^{-1} P)				
	Control	[1]	*NoP*		*Treatment P-value* [2]	*Control*		*NoP*		*Treatment P-value*	*Control*		*NoP*		*Treatment P-value*
bol_037	125.45 ± 50.44	a	56.80 ± 32.03	ab	0.061	18677 ± 5058	a	36463 ± 14062	a	0.055	404 ± 96	ab	835 ± 142	ab	0.002
bol_144	97.78 ± 48.72	a	47.27 ± 8.11	a	0.143	31259 ± 18091	ab	59577 ± 50177	a	0.334	595 ± 237	b	1060 ± 421	b	0.119
eq_973	95.72 ± 39.01	a	44.11 ± 19.13	a	0.055	14572 ± 5050	a	30997 ± 17699	a	0.125	392 ± 21	ab	844 ± 225	ab	0.007
mex_pas	157.00 ± 53.29	a	57.53 ± 21.28	ab	0.013	41136 ± 2866	b	29694 ± 12004	a	0.113	524 ± 68	ab	717 ± 98	ab	0.018
mex_scm	223.69 ± 44.66	a	94.92 ± 39.09	b	0.005	29432 ± 7742	ab	44402 ± 10087	a	0.057	362 ± 37	a	710 ± 155	ab	0.005
mu_esp	189.61 ± 97.19	a	45.18 ± 11.97	a	0.026	33253 ± 7631	ab	19516 ± 6149	a	0.031	438 ± 71	ab	663 ± 130	ab	0.023
sp_bola	148.67 ± 65.36	a	66.21 ± 25.38	ab	0.057	31944 ± 10008	ab	31704 ± 22963	a	0.985	479 ± 107	ab	663 ± 106	ab	0.051
sp_cat	175.84 ± 66.26	a	45.79 ± 13.79	a	0.009	31935 ± 9675	ab	40814 ± 26952	a	0.558	432 ± 57	ab	900 ± 265	ab	0.014
sp_cwr	123.32 ± 14.73	a	47.02 ± 7.36	a	0.000	16575 ± 7431	a	32740 ± 8011	a	0.042	361 ± 73	a	840 ± 159	ab	0.005
sp_mel	120.90 ± 69.75	a	27.60 ± 10.40	a	0.038	16998 ± 9057	a	21241 ± 6183	a	0.469	378 ± 54	ab	897 ± 181	ab	0.002
sp_piq	183.73 ± 56.95	a	59.21 ± 10.62	ab	0.005	26919 ± 12480	ab	33462 ± 11171	a	0.464	375 ± 70	ab	744 ± 107	ab	0.001
usa_chi	164.82 ± 16.31	a	56.12 ± 7.72	ab	0.000	17912 ± 1299	a	14519 ± 3892	a	0.149	330 ± 9	a	505 ± 41	a	0.000
Global mean	151.12 ± 62.65		54.12 ± 23.67			26083 ± 11500		32361 ± 19523			424 ± 111		776 ± 210		

[1] Different letters within columns indicate significant differences among accessions for a given treatment and trait according to Student-Newman-Keuls post-hoc test for $P < 0.05$.

[2] Treatment P-value indicates whether or not treatments are significantly different for a given accession and trait (within lines). The coloured values (red) indicate significant differences between treatments.

Supp. Table 3 (d) - Trial 1 mean values and standard deviation for P tissue concentration traits [P]Tissue (g/100g DW) , P content traits RootP (g), ShootP (g), FruitP (g) and PTP (mg), efficiency parameters PPUE (g^2 DW g^{-1} P) and PER (g DW g^{-1} P), and morphometric traits RW (g), SW (g), BW (g), RSR, LRL (m), RAD (mm), RHW%, PLFR (%), and LRSL (m/g) were considered.

Abbreviation	Root weight (RW, g)					Lateral root weight (LRW, g)					Root hairs weight (RHW, g)				
	Control	[1]	*NoP*		*Treatments P-value* [2]	*Control*		*NoP*		*Treatments P-value*	*Control*		*NoP*		*Treatments P-value*
bol_037	4.09 ± 1.74	ab	3.57 ± 1.49	ab	0.553	2.32 ± 1.10	a-c	2.12 ± 0.81	ab	0.709	1.77 ± 1.06	a	1.44 ± 0.81	ab	0.523
bol_144	6.68 ± 3.77	b	4.88 ± 1.89	bc	0.313	4.85 ± 2.67	c	3.08 ± 1.47	bc	0.177	1.83 ± 1.96	a	1.80 ± 0.95	ab	0.978
eq_973	6.72 ± 3.26	b	5.31 ± 2.29	bc	0.427	4.35 ± 1.65	bc	3.02 ± 1.24	bc	0.161	2.37 ± 1.77	a	2.29 ± 1.11	ab	0.931
mex_pas	5.96 ± 1.38	ab	3.80 ± 1.28	ab	0.006	3.63 ± 1.04	a-c	2.09 ± 0.67	ab	0.003	2.33 ± 0.50	a	1.71 ± 0.76	ab	0.072
mex_scm	6.56 ± 3.22	b	6.61 ± 1.65	c	0.976	3.48 ± 1.79	a-c	3.80 ± 0.95	c	0.685	3.08 ± 1.52	a	2.80 ± 1.23	b	0.715
mu_esp	3.38 ± 0.88	a	1.80 ± 0.47	a	0.002	1.52 ± 0.45	a	1.07 ± 0.42	a	0.084	1.86 ± 0.65	a	0.88 ± 0.39	a	0.011
sp_bola	5.06 ± 1.65	ab	4.06 ± 2.31	a-c	0.345	2.93 ± 0.82	a-c	2.04 ± 0.74	ab	0.048	2.14 ± 0.95	a	2.02 ± 1.73	ab	0.868
sp_cat	5.81 ± 2.70	ab	5.65 ± 1.00	bc	0.903	3.62 ± 2.20	a-c	2.76 ± 0.45	bc	0.419	2.19 ± 0.61	a	2.89 ± 0.57	b	0.102
sp_cwr	4.51 ± 0.63	ab	3.61 ± 0.63	ab	0.044	2.47 ± 0.26	a-c	2.11 ± 0.39	ab	0.099	2.04 ± 0.61	a	1.51 ± 0.28	ab	0.106
sp_mel	5.71 ± 2.84	ab	3.99 ± 1.58	a-c	0.269	3.60 ± 1.62	a-c	2.32 ± 0.95	ab	0.168	2.12 ± 1.30	a	1.67 ± 1.13	ab	0.575
sp_piq	7.03 ± 2.36	b	5.26 ± 1.65	bc	0.115	3.58 ± 1.33	a-c	2.95 ± 0.88	bc	0.299	3.45 ± 1.22	a	2.31 ± 1.03	ab	0.069
usa_chi	4.27 ± 1.56	ab	1.99 ± 0.34	a	0.001	1.98 ± 0.74	ab	0.93 ± 0.21	a	0.002	2.29 ± 0.88	a	1.06 ± 0.25	ab	0.002
Global mean	5.49 ± 2.48		4.15 ± 1.98			3.17 ± 1.64		2.32 ± 1.12			2.32 ± 1.21		1.86 ± 1.07		

[1] Different letters within columns indicate significant differences among accessions for a given treatment and trait according to Student-Newman-Keuls post-hoc test for $P < 0.05$.

[2] Treatment P-value indicates whether or not treatments are significantly different for a given accession and trait (within lines). The coloured values (red) indicate significant differences between treatments.

Supp. Table 3 (e) - Trial 1 mean values and standard deviation for P tissue concentration traits [P]Tissue (g/100g DW) , P content traits RootP (g), ShootP (g), FruitP (g) and PTP (mg), efficiency parameters PPUE (g^2 DW g^{-1} P) and PER (g DW g^{-1} P), and morphometric traits RW (g), SW (g), BW (g), RSR, LRL (m), RAD (mm), RHW%, PLFR (%), and LRSL (m/g) were considered.

Abbreviation	Total biomass (BW, g)					Root to shoot ratio (RSR)					Lateral root length (LRL, m)				
	Control	[1]	*NoP*		*Treatments P-value* [2]	*Control*		*NoP*		*Treatments P-value*	*Control*		*NoP*		*Treatments P-value*
bol_037	39.78 ± 15.89	a-c	31.16 ± 22.95	a	0.430	0.17 ± 0.04	ab	0.22 ± 0.07	ab	0.175	15.64 ± 3.44	a-c	20.85 ± 7.97	ab	0.138
bol_144	36.83 ± 22.89	ab	35.33 ± 21.99	a	0.907	0.27 ± 0.07	c	0.22 ± 0.11	ab	0.403	17.38 ± 5.49	a-c	20.01 ± 7.47	ab	0.531
eq_973	33.82 ± 13.83	a	29.03 ± 16.88	a	0.585	0.27 ± 0.09	c	0.29 ± 0.11	ab	0.768	18.79 ± 10.11	a-c	28.10 ± 5.92	b	0.149
mex_pas	74.32 ± 20.53	d	34.03 ± 12.71	a	0.000	0.18 ± 0.06	a-c	0.24 ± 0.06	ab	0.104	20.59 ± 4.73	bc	19.96 ± 6.84	ab	0.841
mex_scm	58.20 ± 31.54	a-d	50.85 ± 21.21	a	0.619	0.17 ± 0.02	ab	0.20 ± 0.04	ab	0.070	16.89 ± 4.87	a-c	18.38 ± 3.98	ab	0.607
mu_esp	58.21 ± 28.43	a-d	25.71 ± 6.22	a	0.007	0.14 ± 0.03	a	0.15 ± 0.03	a	0.452	9.97 ± 6.09	a	12.65 ± 2.85	a	0.341
sp_bola	56.23 ± 22.71	a-d	33.70 ± 22.89	a	0.079	0.21 ± 0.06	a-c	0.27 ± 0.09	ab	0.146	13.54 ± 6.96	a-c	13.82 ± 5.91	a	0.940
sp_cat	68.25 ± 25.46	cd	39.64 ± 17.74	a	0.073	0.21 ± 0.02	a-c	0.32 ± 0.11	b	0.062	21.83 ± 9.39	c	22.64 ± 3.66	ab	0.876
sp_cwr	43.69 ± 7.86	a-c	38.86 ± 2.71	a	0.225	0.25 ± 0.06	bc	0.23 ± 0.02	ab	0.569	11.96 ± 3.24	ab	19.81 ± 5.42	ab	0.015
sp_mel	43.59 ± 21.57	a-c	22.27 ± 6.93	a	0.069	0.21 ± 0.05	a-c	0.34 ± 0.13	b	0.059	14.35 ± 4.56	a-c	12.85 ± 6.39	a	0.729
sp_piq	64.65 ± 25.29	b-d	39.54 ± 10.67	a	0.028	0.23 ± 0.04	bc	0.27 ± 0.06	ab	0.115	22.40 ± 10.84	c	17.97 ± 5.16	ab	0.343
usa_chi	57.95 ± 25.12	a-d	23.52 ± 7.40	a	0.003	0.21 ± 0.02	a-c	0.26 ± 0.05	ab	0.027	15.00 ± 3.38	a-c	19.18 ± 8.54	ab	0.287
Global mean	53.81 ± 24.82		33.45 ± 16.63			0.21 ± 0.06		0.25 ± 0.08			16.32 ± 7.53		18.74 ± 6.83		

[1] Different letters within columns indicate significant differences among accessions for a given treatment and trait according to Student-Newman-Keuls post-hoc test for $P < 0.05$.

[2] Treatment P-value indicates whether or not treatments are significantly different for a given accession and trait (within lines). The coloured values (red) indicate significant differences between treatments.

Supp. Table 3 (f) - Trial 1 mean values and standard deviation for P tissue concentration traits [P]Tissue (g/100g DW) , P content traits RootP (g), ShootP (g), FruitP (g) and PTP (mg), efficiency parameters PPUE (g^2 DW g^{-1} P) and PER (g DW g^{-1} P), and morphometric traits RW (g), SW (g), BW (g), RSR, LRL (m), RAD (mm), RHW%, PLFR (%), and LRSL (m/g) were considered.

Abbreviation	Lateral root average diameter (LRAD, mm)					Root hairs weight % (RHW%, %)				
	Control	[1]	*NoP*		*Treatments P-value* [2]	*Control*		*NoP*		*Treatments P-value*
bol_037	0.70 ± 0.06	b	0.64 ± 0.13	a-c	0.271	0.42 ± 0.14	bc	0.40 ± 0.11	a	0.690
bol_144	0.75 ± 0.11	b	0.58 ± 0.08	a-c	0.016	0.25 ± 0.16	a	0.37 ± 0.14	a	0.156
eq_973	0.63 ± 0.14	ab	0.53 ± 0.08	a	0.224	0.30 ± 0.15	ab	0.42 ± 0.05	a	0.117
mex_pas	0.74 ± 0.10	b	0.66 ± 0.10	a-c	0.209	0.39 ± 0.05	bc	0.44 ± 0.10	a	0.236
mex_scm	0.66 ± 0.13	ab	0.71 ± 0.05	bc	0.386	0.47 ± 0.05	bc	0.42 ± 0.10	a	0.243
mu_esp	0.57 ± 0.10	ab	0.52 ± 0.04	a	0.268	0.54 ± 0.10	c	0.47 ± 0.11	a	0.255
sp_bola	0.68 ± 0.09	b	0.60 ± 0.06	a-c	0.128	0.41 ± 0.07	bc	0.43 ± 0.16	a	0.850
sp_cat	0.67 ± 0.06	b	0.58 ± 0.06	a-c	0.054	0.41 ± 0.11	bc	0.51 ± 0.02	a	0.075
sp_cwr	0.63 ± 0.08	ab	0.58 ± 0.06	a-c	0.311	0.45 ± 0.09	bc	0.42 ± 0.03	a	0.514
sp_mel	0.66 ± 0.08	ab	0.64 ± 0.07	a-c	0.667	0.36 ± 0.07	a-c	0.40 ± 0.14	a	0.583
sp_piq	0.69 ± 0.09	b	0.72 ± 0.19	c	0.669	0.50 ± 0.09	c	0.42 ± 0.12	a	0.186
usa_chi	0.50 ± 0.04	a	0.57 ± 0.13	ab	0.268	0.54 ± 0.06	c	0.53 ± 0.07	a	0.873
Global mean	0.66 ± 0.11		0.62 ± 0.11			0.42 ± 0.13		0.44 ± 0.11		

[1] Different letters within columns indicate significant differences among accessions for a given treatment and trait according to Student-Newman-Keuls post-hoc test for $P < 0.05$.

[2] Treatment P-value indicates whether or not treatments are significantly different for a given accession and trait (within lines). The coloured values (red) indicate significant differences between treatments.

Supp. Table 3 (g) - Trial 1 mean values and standard deviation for P tissue concentration traits [P]Tissue (g/100g DW) , P content traits RootP (g), ShootP (g), FruitP (g) and PTP (mg), efficiency parameters PPUE (g^2 DW g^{-1} P) and PER (g DW g^{-1} P), and morphometric traits RW (g), SW (g), BW (g), RSR, LRL (m), RAD (mm), RHW%, PLFR (%), and LRSL (m/g) were considered.

Abbreviation	Proportion of root length devoted to fine roots (PLFR, %)					Lateral root specific length (LRSL, m/g)				
	Control [1]		*NoP*		*Treatments P-value* [2]	*Control*		*NoP*		*Treatments P-value*
bol_037	0.82 ± 0.04	b	0.87 ± 0.06	ab	0.154	7.51 ± 2.41	b	10.38 ± 3.35	ab	0.091
bol_144	0.79 ± 0.05	b	0.86 ± 0.04	ab	0.023	4.00 ± 0.68	ab	8.52 ± 7.39	ab	0.210
eq_973	0.86 ± 0.07	b	0.91 ± 0.03	ab	0.196	4.97 ± 1.37	ab	8.86 ± 4.11	ab	0.089
mex_pas	0.81 ± 0.05	b	0.86 ± 0.06	ab	0.106	5.57 ± 1.48	ab	9.52 ± 1.93	ab	0.002
mex_scm	0.82 ± 0.07	b	0.83 ± 0.04	ab	0.711	4.40 ± 0.81	a	5.12 ± 0.96	a	0.256
mu_esp	0.87 ± 0.05	b	0.93 ± 0.03	b	0.014	6.34 ± 2.66	ab	13.66 ± 1.70	ab	0.001
sp_bola	0.82 ± 0.04	b	0.89 ± 0.03	ab	0.009	4.51 ± 1.69	ab	7.12 ± 1.49	a	0.018
sp_cat	0.83 ± 0.03	b	0.88 ± 0.02	ab	0.019	6.62 ± 1.57	ab	7.96 ± 0.91	a	0.176
sp_cwr	0.85 ± 0.02	b	0.88 ± 0.03	ab	0.054	4.91 ± 1.43	ab	9.37 ± 1.67	ab	0.001
sp_mel	0.83 ± 0.02	b	0.85 ± 0.04	ab	0.322	3.97 ± 1.79	ab	6.14 ± 1.83	a	0.176
sp_piq	0.80 ± 0.05	b	0.81 ± 0.09	a	0.842	5.91 ± 1.76	ab	6.32 ± 1.88	a	0.674
usa_chi	0.92 ± 0.02	a	0.91 ± 0.08	ab	0.717	8.10 ± 2.41	ab	19.66 ± 5.57	b	0.001
Global mean	0.83 ± 0.05		0.87 ± 0.06			5.72 ± 2.13		9.60 ± 5.21		

[1] Different letters within columns indicate significant differences among accessions for a given treatment and trait according to Student-Newman-Keuls post-hoc test for $P < 0.05$.

[2] Treatment P-value indicates whether or not treatments are significantly different for a given accession and trait (within lines). The coloured values (red) indicate significant differences between treatments.

Supp. Table 4 (a) - Trial 2 mean values and standard deviation for P concentration trait [P]Shoot (g/100g DW), P content trait PTP (mg), efficiency parameters PPUE (g^2 DW g^{-1} P) and PER (g DW g^{-1} P), and morphometric traits RW (g), SW (g), BW (g), RSR, RTL (m), TAD (mm), RHW%, PLF (%), and RSL (m/g) were considered.

Abbreviation	[P]Shoot (g/100g DW)					Plant total P content (PTP, mg)				
	Control	[1]	*NoP*		*Treatment P-value* [2]	*Control*		*NoP*		*Treatment P-value*
fra_DLL	0.43 ± 0.04	a	0.29 ± 0.04	a	0.0008	183.53 ± 44.37	a	106.21 ± 22.74	ab	0.0085
mex_096D	0.86 ± 0.08	c	0.48 ± 0.11	bc	0.0001	527.81 ± 146.49	ab	134.40 ± 75.58	ab	0.0002
mex_103B	0.66 ± 0.09	bc	0.49 ± 0.07	bc	0.0050	528.40 ± 175.71	ab	167.21 ± 58.92	b	0.0008
mex_ng	0.70 ± 0.17	bc	0.43 ± 0.11	b	0.0109	538.51 ± 257.44	ab	104.60 ± 68.73	ab	0.0031
mex_pas	0.50 ± 0.05	ab	0.44 ± 0.07	b	0.1139	316.75 ± 104.42	ab	143.45 ± 49.89	ab	0.0043
mex_scm	0.71 ± 0.12	bc	0.53 ± 0.06	bc	0.0053	504.93 ± 291.63	ab	79.98 ± 34.19	ab	0.0059
mu_esp	0.67 ± 0.12	bc	0.48 ± 0.09	bc	0.0107	291.09 ± 151.79	ab	68.25 ± 30.30	a	0.0055
sp_060	0.84 ± 0.14	c	0.64 ± 0.13	c	0.0351	303.40 ± 94.35	ab	130.47 ± 39.41	ab	0.0020
sp_11814	0.67 ± 0.18	bc	0.49 ± 0.08	bc	0.0466	319.76 ± 116.67	ab	106.16 ± 52.77	ab	0.0022
sp_anc	0.73 ± 0.13	bc	0.47 ± 0.06	b	0.0014	446.20 ± 133.98	ab	147.78 ± 33.10	ab	0.0003
sp_bola	0.65 ± 0.12	bc	0.46 ± 0.10	b	0.0132	388.35 ± 127.33	ab	82.23 ± 34.18	ab	0.0002
sp_lam	0.73 ± 0.13	bc	0.51 ± 0.05	bc	0.0054	200.85 ± 62.09	a	127.13 ± 48.09	ab	0.0588
sp_lobo	0.69 ± 0.18	bc	0.52 ± 0.10	bc	0.0776	177.72 ± 103.33	a	114.97 ± 16.83	ab	0.1730
sp_piq	0.75 ± 0.10	c	0.43 ± 0.18	b	0.0037	484.97 ± 221.40	ab	63.41 ± 14.42	a	0.0009
usa_conq	0.76 ± 0.13	c	0.46 ± 0.05	b	0.0004	634.93 ± 386.93	b	128.67 ± 37.11	ab	0.0096
usa_jap	0.67 ± 0.09	bc	0.41 ± 0.09	b	0.0006	277.82 ± 142.06	ab	141.63 ± 25.31	ab	0.0434
usa_numex	0.83 ± 0.11	c	0.55 ± 0.08	bc	0.0004	438.88 ± 145.96	ab	154.16 ± 49.43	ab	0.0011
usa_sandia	0.72 ± 0.06	bc	0.46 ± 0.06	b	0.0000	905.36 ± 472.53	c	109.75 ± 50.74	ab	0.0025
Global mean	0.70 ± 0.15		0.48 ± 0.11			412.68 ± 252.55		117.26 ± 50.24		

[1] Different letters within columns indicate significant differences among accessions for a given treatment and trait according to Student-Newman-Keuls post-hoc test for $P < 0.05$.

[2] Treatment P-value indicates whether or not treatments are significantly different for a given accession and trait (within lines). The coloured values (red) indicate significant differences between treatments.

Supp. Table 4 (b) - Trial 2 mean values and standard deviation for P concentration trait [P]Shoot (g/100g DW), P content trait PTP (mg), efficiency parameters PPUE (g^2 DW g^{-1} P) and PER (g DW g^{-1} P), and morphometric traits RW (g), SW (g), BW (g), RSR, RTL (m), TAD (mm), RHW%, PLF (%), and RSL (m/g) were considered.

Abbreviation	Physiological P use efficiency (PPUE, g^2 DW g^{-1} P)					P efficiency ratio (PER, g DW g^{-1} P)				
	Control	[1]	*NoP*		*Treatment P-value* [2]	*Control*		*NoP*		*Treatment P-value*
fra_DLL	10788 ± 2147	ab	13567 ± 1858	c	0.0601	236 ± 21.06	c	351 ± 41.88	c	0.0006
mex_096D	7466 ± 2085	a	8123 ± 8624	ab	0.8597	117 ± 10.99	a	220 ± 59.99	ab	0.0021
mex_103B	13728 ± 5227	ab	7907 ± 3122	ab	0.0412	155 ± 20.06	a	208 ± 28.17	ab	0.0039
mex_ng	13299 ± 10213	ab	6180 ± 4393	ab	0.1541	148 ± 32.92	a	247 ± 67.76	b	0.0163
mex_pas	13100 ± 3090	ab	7902 ± 2647	ab	0.0107	200 ± 17.58	b	230 ± 33.64	ab	0.0867
mex_scm	10768 ± 5735	ab	2935 ± 872	a	0.0087	145 ± 24.63	a	190 ± 24.40	ab	0.0143
mu_esp	7068 ± 5060	a	3822 ± 2680	ab	0.1951	153 ± 27.68	a	216 ± 39.89	ab	0.0100
sp_060	4622 ± 1009	a	3705 ± 1802	ab	0.3022	122 ± 20.76	a	160 ± 29.51	a	0.0281
sp_11814	8175 ± 3483	a	4855 ± 2484	ab	0.0865	156 ± 32.57	a	209 ± 33.79	ab	0.0190
sp_anc	9401 ± 3690	ab	7486 ± 2186	ab	0.2997	141 ± 24.98	a	217 ± 27.32	ab	0.0005
sp_bola	10018 ± 2345	ab	4099 ± 698	ab	0.0001	158 ± 28.29	a	224 ± 47.23	ab	0.0152
sp_lam	4095 ± 868	a	5288 ± 1804	ab	0.1830	140 ± 25.85	a	200 ± 20.16	ab	0.0023
sp_lobo	4314 ± 2168	a	5036 ± 1714	ab	0.5517	151 ± 33.10	a	198 ± 37.97	ab	0.0577
sp_piq	9332 ± 4079	ab	4596 ± 1922	ab	0.0278	135 ± 18.40	a	258 ± 82.73	b	0.0052
usa_conq	12634 ± 8625	ab	6684 ± 2546	ab	0.1362	135 ± 21.56	a	217 ± 22.88	ab	0.0001
usa_jap	6802 ± 3923	a	9865 ± 4071	bc	0.2139	152 ± 22.86	a	256 ± 51.59	b	0.0011
usa_numex	6753 ± 1036	a	5591 ± 2097	ab	0.2514	123 ± 14.41	a	185 ± 23.32	ab	0.0003
usa_sandia	17345 ± 6738	b	5907 ± 3407	ab	0.0052	139 ± 12.98	a	220 ± 25.75	ab	0.0001
Global mean	9337 ± 5474		6249 ± 3834			150 ± 34.32		222 ± 54.00		

[1] Different letters within columns indicate significant differences among accessions for a given treatment and trait according to Student-Newman-Keuls post-hoc test for $P < 0.05$.

[2] Treatment P-value indicates whether or not treatments are significantly different for a given accession and trait (within lines). The coloured values (red) indicate significant differences between treatments.

Supp. Table 4 (c) - Trial 2 mean values and standard deviation for P concentration trait [P]Shoot (g/100g DW), P content trait PTP (mg), efficiency parameters PPUE (g^2 DW g^{-1} P) and PER (g DW g^{-1} P), and morphometric traits RW (g), SW (g), BW (g), RSR, RTL (m), TAD (mm), RHW%, PLF (%), and RSL (m/g) were considered.

Abbreviation	Root weight (RW, g)					Lateral root weight (LRW, g)					Root hairs weight (RHW, g)				
	Control	[1]	*NoP*		*Treatment P-value* [2]	*Control*		*NoP*		*Treatment P-value*	*Control*		*NoP*		*Treatment P-value*
fra_DLL	3.89 ± 1.36	a-c	2.68 ± 0.58	b	0.1530	0.92 ± 0.38	ab	0.73 ± 0.18	ab	0.3979	2.97 ± 1.00	a-c	1.95 ± 0.69	b	0.1461
mex_096D	3.15 ± 0.45	a	1.81 ± 1.03	ab	0.0148	1.19 ± 0.27	ab	0.84 ± 0.33	ab	0.0731	1.96 ± 0.36	ab	0.97 ± 0.75	ab	0.0144
mex_103B	6.47 ± 2.66	c	2.33 ± 0.23	ab	0.0035	2.59 ± 0.78	c	1.11 ± 0.30	b	0.0015	3.88 ± 1.97	a-c	1.23 ± 0.33	ab	0.0085
mex_ng	6.27 ± 2.33	bc	1.87 ± 0.95	ab	0.0021	2.50 ± 1.09	c	0.78 ± 0.47	ab	0.0066	3.77 ± 1.31	a-c	1.08 ± 0.70	ab	0.0018
mex_pas	3.83 ± 1.33	a-c	2.09 ± 0.38	ab	0.0115	1.78 ± 0.44	b	1.12 ± 0.12	b	0.0051	2.05 ± 1.00	ab	0.97 ± 0.33	ab	0.0315
mex_scm	3.92 ± 2.34	a-c	1.13 ± 0.18	a	0.0165	1.26 ± 0.44	ab	0.59 ± 0.16	ab	0.0170	2.66 ± 1.97	a-c	0.54 ± 0.11	a	0.0262
mu_esp	2.35 ± 1.00	a	0.98 ± 0.43	a	0.0136	0.64 ± 0.19	a	0.40 ± 0.16	a	0.0509	1.71 ± 0.87	a	0.57 ± 0.31	a	0.0148
sp_060	4.08 ± 1.11	a-c	1.70 ± 0.61	ab	0.0022	0.84 ± 0.30	ab	0.57 ± 0.23	ab	0.1346	3.24 ± 1.04	a-c	1.14 ± 0.68	ab	0.0044
sp_11814	4.32 ± 1.18	a-c	1.98 ± 1.13	ab	0.0084	0.85 ± 0.27	ab	0.77 ± 0.55	ab	0.7645	3.46 ± 1.11	a-c	1.20 ± 0.63	ab	0.0021
sp_anc	4.16 ± 1.95	a-c	2.17 ± 0.49	ab	0.0373	1.38 ± 0.44	ab	0.87 ± 0.20	ab	0.0313	2.78 ± 1.61	a-c	1.30 ± 0.35	ab	0.0540
sp_bola	4.96 ± 1.63	a-c	1.80 ± 1.00	ab	0.0023	0.70 ± 0.23	ab	0.66 ± 0.29	ab	0.7789	4.26 ± 1.72	bc	1.15 ± 0.74	ab	0.0022
sp_lam	2.44 ± 0.68	a	1.56 ± 0.51	ab	0.0596	0.87 ± 0.10	ab	0.66 ± 0.17	ab	0.0382	1.57 ± 0.62	a	0.90 ± 0.37	ab	0.0947
sp_lobo	2.55 ± 1.47	a	2.21 ± 0.61	ab	0.6194	0.75 ± 0.52	ab	0.68 ± 0.33	ab	0.8032	1.80 ± 0.98	a	1.53 ± 0.29	ab	0.5275
sp_piq	6.15 ± 2.87	bc	1.70 ± 0.43	ab	0.0038	1.53 ± 0.50	ab	0.74 ± 0.23	ab	0.0052	4.62 ± 2.89	c	0.96 ± 0.37	ab	0.0117
usa_conq	5.95 ± 3.19	bc	2.42 ± 0.86	ab	0.0258	1.46 ± 0.57	ab	0.86 ± 0.44	ab	0.0700	4.50 ± 2.69	c	1.57 ± 0.88	ab	0.0296
usa_jap	2.49 ± 1.40	a	1.27 ± 0.22	ab	0.0620	0.79 ± 0.47	ab	0.60 ± 0.17	ab	0.3872	1.70 ± 1.03	a	0.67 ± 0.11	a	0.0347
usa_numex	3.46 ± 0.75	a-c	1.74 ± 0.61	ab	0.0014	0.78 ± 0.34	ab	0.48 ± 0.20	a	0.0946	2.69 ± 0.51	a-c	1.26 ± 0.50	ab	0.0006
usa_sandia	6.04 ± 2.65	bc	2.03 ± 0.88	ab	0.0078	1.19 ± 0.63	ab	0.49 ± 0.13	a	0.0267	4.84 ± 2.07	c	1.54 ± 0.84	ab	0.0073
Global mean	4.28 ± 2.20		1.85 ± 0.76			1.25 ± 0.73		0.72 ± 0.33			3.03 ± 1.79		1.13 ± 0.61		

[1] Different letters within columns indicate significant differences among accessions for a given treatment and trait according to Student-Newman-Keuls post-hoc test for $P < 0.05$.

[2] Treatment P-value indicates whether or not treatments are significantly different for a given accession and trait (within lines). The coloured values (red) indicate significant differences between treatments.

Supp. Table 4 (d) - Trial 2 mean values and standard deviation for P concentration trait [P]Shoot (g/100g DW), P content trait PTP (mg), efficiency parameters PPUE (g^2 DW g^{-1} P) and PER (g DW g^{-1} P), and morphometric traits RW (g), SW (g), BW (g), RSR, RTL (m), TAD (mm), RHW%, PLF (%), and RSL (m/g) were considered.

Abbreviation	Shoot weight (SW, g)					Total biomass (BW, g)					Root to shot ratio (RSR)				
	Control	[1]	*NoP*		*Treatment P-value* [2]	*Control*		*NoP*		*Treatment P-value*	*Control*		*NoP*		*Treatment P-value*
fra_DLL	42.80 ± 9.09	ab	36.60 ± 4.10	e	0.2020	45.91 ± 8.71	ab	38.75 ± 3.44	d	0.1253	0.10 ± 0.03	ab	0.08 ± 0.01	ab	0.2346
mex_096D	61.00 ± 16.35	ab	31.00 ± 23.87	b-e	0.0293	64.15 ± 16.77	ab	32.81 ± 24.86	b-d	0.0283	0.06 ± 0.01	a	0.07 ± 0.03	ab	0.2512
mex_103B	81.33 ± 26.13	a-c	35.00 ± 12.90	c-e	0.0030	87.81 ± 28.55	a-c	37.33 ± 13.02	cd	0.0028	0.08 ± 0.01	ab	0.08 ± 0.03	ab	0.7847
mex_ng	79.80 ± 48.65	a-c	24.17 ± 16.69	a-e	0.0267	86.07 ± 50.85	a-c	26.03 ± 17.62	a-d	0.0233	0.09 ± 0.03	ab	0.10 ± 0.04	b	0.7319
mex_pas	62.33 ± 17.05	ab	32.33 ± 10.37	c-e	0.0042	66.16 ± 18.18	ab	34.42 ± 10.40	b-d	0.0040	0.06 ± 0.01	ab	0.07 ± 0.03	ab	0.5871
mex_scm	71.20 ± 39.00	ab	14.67 ± 5.28	a	0.0062	75.12 ± 41.33	ab	15.80 ± 5.36	a	0.0065	0.06 ± 0.01	a	0.09 ± 0.06	ab	0.2093
mu_esp	43.83 ± 26.33	ab	15.50 ± 9.01	a	0.0318	45.79 ± 27.27	ab	16.48 ± 9.27	a	0.0318	0.06 ± 0.02	a	0.08 ± 0.03	ab	0.2982
sp_060	35.67 ± 7.37	ab	20.83 ± 7.63	a-c	0.0065	38.39 ± 8.83	ab	22.53 ± 8.21	a-d	0.0092	0.11 ± 0.01	b	0.08 ± 0.01	ab	0.0084
sp_11814	48.17 ± 15.30	ab	21.50 ± 10.48	a-d	0.0055	51.77 ± 15.49	ab	23.47 ± 11.48	a-c	0.0049	0.10 ± 0.01	ab	0.09 ± 0.02	ab	0.6301
sp_anc	62.33 ± 20.48	ab	32.00 ± 7.80	c-e	0.0069	65.80 ± 21.96	ab	34.16 ± 7.96	b-d	0.0078	0.07 ± 0.02	ab	0.07 ± 0.02	ab	0.8665
sp_bola	59.00 ± 12.57	ab	17.17 ± 4.36	ab	0.0000	63.96 ± 13.14	ab	18.97 ± 4.77	ab	0.0000	0.09 ± 0.03	ab	0.11 ± 0.05	b	0.3901
sp_lam	27.17 ± 6.05	a	25.20 ± 8.53	a-e	0.6649	29.61 ± 6.50	a	26.45 ± 8.97	a-d	0.5153	0.09 ± 0.02	ab	0.06 ± 0.01	ab	0.0417
sp_lobo	26.40 ± 13.72	a	22.67 ± 4.72	a-e	0.5449	27.93 ± 13.93	a	24.87 ± 4.59	a-d	0.6220	0.10 ± 0.03	ab	0.10 ± 0.04	b	0.8044
sp_piq	64.00 ± 29.04	ab	15.50 ± 3.08	a	0.0023	70.15 ± 30.78	ab	17.20 ± 3.43	a	0.0019	0.10 ± 0.04	ab	0.11 ± 0.02	b	0.6309
usa_conq	86.17 ± 57.00	bc	28.00 ± 8.92	a-e	0.0331	92.12 ± 58.65	bc	30.43 ± 9.68	a-d	0.0293	0.08 ± 0.03	ab	0.09 ± 0.02	ab	0.5047
usa_jap	41.83 ± 21.69	ab	36.17 ± 9.89	de	0.5733	44.33 ± 22.59	ab	37.44 ± 10.08	cd	0.5110	0.06 ± 0.02	ab	0.03 ± 0.01	a	0.0102
usa_numex	52.33 ± 12.11	ab	28.33 ± 9.71	a-e	0.0036	55.80 ± 11.74	ab	30.07 ± 10.14	a-d	0.0023	0.07 ± 0.03	ab	0.06 ± 0.02	ab	0.6145
usa_sandia	122.60 ± 56.24	c	24.33 ± 12.63	a-e	0.0023	127.43 ± 57.84	c	26.36 ± 13.47	a-d	0.0023	0.06 ± 0.02	a	0.09 ± 0.03	ab	0.0599
Global mean	58.88 ± 34.01		25.51 ± 12.08			62.79 ± 35.56		27.32 ± 12.54			0.08 ± 0.03		0.08 ± 0.03		

[1] Different letters within columns indicate significant differences among accessions for a given treatment and trait according to Student-Newman-Keuls post-hoc test for $P < 0.05$.

[2] Treatment P-value indicates whether or not treatments are significantly different for a given accession and trait (within lines). The coloured values (red) indicate significant differences between treatments.

Supp. Table 4 (e) - Trial 2 mean values and standard deviation for P concentration trait [P]Shoot (g/100g DW), P content trait PTP (mg), efficiency parameters PPUE (g^2 DW g^{-1} P) and PER (g DW g^{-1} P), and morphometric traits RW (g), SW (g), BW (g), RSR, RTL (m), TAD (mm), RHW%, PLF (%), and RSL (m/g) were considered.

Abbreviation	Root total length (RTL, m)					Total root average diameter (TAD, mm)				
	Control	[1]	*NoP*		*Treatment P-value* [2]	*Control*		*NoP*		*Treatment P-value*
fra_DLL	3.99 ± 1.41	a-c	4.24 ± 1.89	a-c	0.8394	0.71 ± 0.07	a	0.74 ± 0.02	ab	0.4258
mex_096D	4.83 ± 2.59	a-c	4.80 ± 2.72	a-c	0.9831	0.84 ± 0.09	b-d	0.87 ± 0.05	d	0.5728
mex_103B	6.59 ± 1.35	c	5.90 ± 1.78	bc	0.4678	0.88 ± 0.06	d	0.82 ± 0.02	a-d	0.0244
mex_ng	5.89 ± 1.97	bc	5.89 ± 2.69	bc	0.9980	0.84 ± 0.05	b-d	0.73 ± 0.02	ab	0.0007
mex_pas	5.76 ± 1.32	bc	6.12 ± 1.12	c	0.6244	0.84 ± 0.10	b-d	0.83 ± 0.05	b-c	0.8823
mex_scm	4.38 ± 1.38	a-c	3.70 ± 1.73	a-c	0.4990	0.78 ± 0.06	a-d	0.78 ± 0.06	a-c	0.8764
mu_esp	1.70 ± 0.54	a	3.12 ± 1.57	a-c	0.0875	0.80 ± 0.03	a-d	0.74 ± 0.04	ab	0.0141
sp_060	2.54 ± 0.24	ab	2.99 ± 1.33	ab	0.5267	0.88 ± 0.10	cd	0.86 ± 0.09	cd	0.8357
sp_11814	2.69 ± 1.00	ab	5.66 ± 4.95	bc	0.2240	0.78 ± 0.10	a-d	0.77 ± 0.07	a-c	0.8953
sp_anc	5.94 ± 2.88	bc	5.36 ± 1.32	bc	0.6660	0.79 ± 0.09	a-d	0.75 ± 0.04	ab	0.3217
sp_bola	3.40 ± 1.85	a-c	5.19 ± 2.76	bc	0.2159	0.75 ± 0.07	a-c	0.73 ± 0.04	ab	0.5200
sp_lam	4.70 ± 1.81	a-c	4.34 ± 0.76	a-c	0.7183	0.78 ± 0.04	a-d	0.80 ± 0.07	a-d	0.6112
sp_lobo	3.94 ± 1.44	a-c	4.76 ± 1.93	a-c	0.5048	0.82 ± 0.10	a-d	0.76 ± 0.05	ab	0.2350
sp_piq	5.49 ± 2.23	bc	5.75 ± 1.46	bc	0.8216	0.76 ± 0.09	a-c	0.78 ± 0.04	a-d	0.5293
usa_conq	4.60 ± 2.21	a-c	4.77 ± 2.48	a-c	0.9017	0.79 ± 0.12	a-d	0.73 ± 0.03	a	0.2490
usa_jap	4.12 ± 0.60	a-c	4.50 ± 1.67	a-c	0.6200	0.75 ± 0.09	a-c	0.75 ± 0.04	ab	0.9328
usa_numex	3.34 ± 0.94	a-c	2.03 ± 0.99	a	0.0397	0.72 ± 0.05	ab	0.79 ± 0.05	a-d	0.0509
usa_sandia	3.26 ± 0.68	a-c	3.61 ± 1.07	a-c	0.5864	0.74 ± 0.13	ab	0.75 ± 0.04	ab	0.8088
Global mean	4.37 ± 1.99		4.61 ± 2.27			0.79 ± 0.09		0.78 ± 0.06		

[1] Different letters within columns indicate significant differences among accessions for a given treatment and trait according to Student-Newman-Keuls post-hoc test for $P < 0.05$.

[2] Treatment P-value indicates whether or not treatments are significantly different for a given accession and trait (within lines). The coloured values (red) indicate significant differences between treatments.

Supp. Table 4 (f) - Trial 2 mean values and standard deviation for P concentration trait [P]Shoot (g/100g DW), P content trait PTP (mg), efficiency parameters PPUE (g^2 DW g^{-1} P) and PER (g DW g^{-1} P), and morphometric traits RW (g), SW (g), BW (g), RSR, RTL (m), TAD (mm), RHW%, PLF (%), and RSL (m/g) were considered.

Abbreviation	Root hairs weight % (RHW, %)					Total proportion of root length devoted to fine roots (PFR, %)					Root specific length (RSL, m/g)				
	Control	[1]	***NoP***		***Treatment P-value*** [2]	***Control***		***NoP***		***Treatment P-value***	***Control***		***NoP***		***Treatment P-value***
fra_DLL	0.77 ± 0.04	bc	0.72 ± 0.10	b	0.378	0.82 ± 0.03	b	0.81 ± 0.02	c	0.900	1.05 ± 0.29	a-c	1.66 ± 0.87	ab	0.233
mex_096D	0.62 ± 0.06	ab	0.50 ± 0.09	ab	0.021	0.74 ± 0.06	ab	0.73 ± 0.05	a	0.547	1.53 ± 0.72	a-d	2.63 ± 0.65	ab	0.020
mex_103B	0.58 ± 0.06	ab	0.52 ± 0.12	ab	0.306	0.73 ± 0.03	ab	0.78 ± 0.02	a-c	0.008	1.09 ± 0.23	a-c	2.53 ± 0.70	ab	0.001
mex_ng	0.61 ± 0.06	ab	0.56 ± 0.14	ab	0.521	0.76 ± 0.02	ab	0.83 ± 0.01	c	0.000	0.98 ± 0.32	ab	3.22 ± 0.50	b	0.000
mex_pas	0.51 ± 0.10	a	0.45 ± 0.08	a	0.294	0.73 ± 0.05	ab	0.74 ± 0.05	ab	0.665	1.65 ± 0.60	a-d	3.00 ± 0.72	ab	0.006
mex_scm	0.62 ± 0.16	ab	0.48 ± 0.09	a	0.109	0.78 ± 0.03	ab	0.80 ± 0.04	c	0.425	1.63 ± 1.41	a-d	3.20 ± 1.17	b	0.074
mu_esp	0.68 ± 0.19	a-c	0.57 ± 0.07	ab	0.208	0.78 ± 0.02	ab	0.83 ± 0.03	c	0.014	0.83 ± 0.38	ab	3.27 ± 1.02	b	0.001
sp_060	0.78 ± 0.08	bc	0.64 ± 0.17	ab	0.150	0.69 ± 0.06	a	0.73 ± 0.06	a	0.332	0.66 ± 0.20	a	1.86 ± 0.68	ab	0.010
sp_11814	0.80 ± 0.08	bc	0.63 ± 0.10	ab	0.013	0.78 ± 0.05	ab	0.81 ± 0.04	c	0.368	0.65 ± 0.31	a	2.48 ± 1.12	ab	0.007
sp_anc	0.64 ± 0.11	a-c	0.60 ± 0.06	ab	0.376	0.77 ± 0.05	ab	0.82 ± 0.02	c	0.061	1.75 ± 1.29	b-d	2.54 ± 0.75	ab	0.239
sp_bola	0.84 ± 0.08	c	0.62 ± 0.09	ab	0.001	0.79 ± 0.04	ab	0.83 ± 0.03	c	0.124	0.82 ± 0.56	ab	2.95 ± 1.21	ab	0.003
sp_lam	0.62 ± 0.08	ab	0.57 ± 0.06	ab	0.298	0.77 ± 0.03	ab	0.78 ± 0.06	a-c	0.955	2.14 ± 1.14	d	2.97 ± 0.83	ab	0.250
sp_lobo	0.71 ± 0.07	a-c	0.71 ± 0.06	b	0.877	0.74 ± 0.06	ab	0.81 ± 0.03	c	0.043	1.67 ± 0.35	a-d	2.12 ± 0.56	ab	0.245
sp_piq	0.71 ± 0.12	a-c	0.56 ± 0.11	ab	0.039	0.78 ± 0.04	ab	0.80 ± 0.02	bc	0.394	1.12 ± 0.65	a-d	3.58 ± 1.23	b	0.002
usa_conq	0.74 ± 0.06	bc	0.63 ± 0.19	ab	0.223	0.77 ± 0.08	ab	0.83 ± 0.01	c	0.071	0.88 ± 0.44	ab	1.97 ± 0.74	ab	0.012
usa_jap	0.67 ± 0.15	a-c	0.53 ± 0.08	ab	0.070	0.82 ± 0.04	b	0.82 ± 0.02	c	1.000	2.06 ± 1.00	cd	3.55 ± 1.26	b	0.046
usa_numex	0.78 ± 0.06	bc	0.72 ± 0.09	b	0.213	0.81 ± 0.03	b	0.78 ± 0.04	a-c	0.198	0.98 ± 0.21	ab	1.19 ± 0.48	a	0.346
usa_sandia	0.80 ± 0.06	bc	0.72 ± 0.13	b	0.261	0.81 ± 0.07	b	0.81 ± 0.03	c	0.979	0.63 ± 0.27	a	2.29 ± 1.65	ab	0.087
Global mean	0.69 ± 0.13		0.59 ± 0.13			0.77 ± 0.05		0.80 ± 0.05			1.25 ± 0.80		2.62 ± 1.09		

[1] Different letters within columns indicate significant differences among accessions for a given treatment and trait according to Student-Newman-Keuls post-hoc test for $P < 0.05$.

[2] Treatment P-value indicates whether or not treatments are significantly different for a given accession and trait (within lines). The coloured values (red) indicate significant differences between treatments.

General discussion |

During the years of *The Green Revolution* we avoided what could have been a catastrophic food shortage, in the developing world, due to a dramatic inability to satisfy the needs of a growing population combined with the exhaustion of arable land where to expand cultivation fields to, in order to increase food production (Khush, 2002). Just a few decades later, we are again facing a similar situation and wondering how can the world's agriculture double its food production before the year 2050, in order to feed a population of 9.2 billion people, while under climate change conditions (Grafton et al., 2015). Hence, agriculture will need a completely new set of approaches, in order to face the advent adversities (Raza et al., 2019).

On that matter, chemical fertilizer applications, particularly nitrate and phosphate, played a major role in increasing food production during *The Green Revolution* (Khush, 2002). However, researchers believe that approach will not produce the same response on today's crops because of diminishing returns, which imply that increasing amounts of fertilizer will not necessarily translate into higher yields (Tilman et al., 2002). Plus, since then, modern agriculture and modern varieties became greatly dependent on chemical fertilizers (Fita et al., 2015). Likewise, arable land is a scarce resource, and today, many cultivated soils are in serious risk of erosion, completely depleted or heavily contaminated, as a result of several years of intensive cultivation, combined with the lack of proper agronomic practices (Mogollón et al., 2018). Thus, the only soils left where to expand, without reducing even more the forested and natural reserve areas, are marginal soils, where it would be extremely difficult to significantly increase yields (Tilman et al., 2002). Furthermore, water is an extremely important resource to agriculture, and is already scarce in many regions of the globe and, on top of that, climate change will intensify, even more, this paradigm (Raza et al., 2019). In addition, with increasing number and size of urban areas, water is being dedicated for other purposes other than agriculture, which makes it an even more scarce resource (Fita et al., 2015; Tilman et al., 2002). Overall, mankind must find a way to increase food production without increasing the toll on the environment and without increasing the already high inputs in agriculture (Grafton et al., 2015).

Hence, researchers will have to increase yields in the already existent production areas of the world. In this regard, the development of highly resilient varieties to biotic and abiotic stresses, as well as, adaptation to low input conditions, for both water and fertilizers, could help mankind to boost food production and, at the same time, help to reduce our footprint on the environment (Fita et al., 2015; Raza et al., 2019). An extremely important resource for crop breeding is the availability of sources of variation (germplasm), which enables breeders to look for advantageous allelic combinations and to select those individuals with higher capability of withstanding harsh conditions to be used in breeding programs or introduced directly into the market. On that regard, related species, wild and semi-domesticated forms are an important source of genetic diversity, since they are often adapted to a wider range of conditions, compared to the cultivated

ones, and maintain a wider genetic diversity (Prohens et al., 2017; Zonneveld et al., 2015). In fact, researchers have resorted to crop wild related species in several occasions in order to introgress traits of interest into other varieties, demonstrating the huge utility of these materials in plant breeding (Chhapekar et al., 2018; Prohens et al., 2017; Wang and Bosland, 2006). This is particularly common for introgression of resistance genes into cultivars (Soler and Nuez, 2004; Yoon et al., 2006).

However, crop wild relatives' application in crop breeding is not always an option or, at least, not an easy way to improve cultivated varieties (Hajjar and Hodgkin, 2007). Thus, wild relatives, especially those outside the primary gene pool, often show strong incompatibility barriers for hybridisation, which seriously compromises the viability of the resulting progeny. In pepper, crosses among species within the *annuum* complex produce fertile hybrids without any assisted hybridisation techniques (Baral and Bosland, 2004; Walsh and Hoot, 2001). Notwithstanding, crossability among species from *annuum*, *baccatum* and *pubescens* complexes are challenging and only possible through assisted hybridisation techniques (Manzur et al., 2015; Onus and Pickersgill, 2004; Zijlstra et al., 1991). In addition, wild relatives are usually linked to undesirable traits, which are vital to their survival in their natural habitats but constitute a negative aspect from an agronomic and commercial point of view. Among those undesirable traits we find lack of adaptation to cultivation systems (Rodríguez-Burruezo et al., 2009), high content in secondary metabolites, vital for plant's defense mechanism but, often with negative repercussions in the fruit's flavour (Luna-Ruiz et al., 2018), prickliness (Plazas et al., 2016), low yields (Hajjar and Hodgkin, 2007), among others. Furthermore, the introgression of wild relatives' genes has a significant linkage drag associated, due to the low recombination, which makes it extremely difficult to dissect the genic region responsible for traits of interest (Prohens et al., 2017). Plus, these traits are often controlled by several genes with dominant nature, which may result in manifestation of undesired phenotype in the progenies (Hajjar and Hodgkin, 2007).

Hence, researchers need other sources from where to mine for variation that do not show the same limitations as the wild specimens. On that matter, traditional varieties, landraces, ecotypes and heirlooms appear as a remarkable source of variation, as opposed to modern varieties, which encompass a narrower genetic diversity (Fita et al., 2015; Hammer and Khoshbakht, 2005; Parisi et al., 2017). Traditional materials are the result of long evolutive and selective process, shaped by local climatic conditions, soil properties, horticultural practices and even pathogens and, therefore, these materials are much more resilient to climate change and more prone to prevail under low input conditions (Fita et al., 2015; Muñoz-Falcón et al., 2008; Parisi et al., 2017; Rivera et al., 2016).

On that matter, Spain is a relevant centre of diversity for *C. annuum*, as a result of a massive introduction of germplasm from the New World (Andrews, 1995; Nuez et al., 2003). More than five-hundred years of selection created a myriad of traditional

varieties, landraces, ecotypes and heirlooms across the country's diverse landscape (Rodríguez-Burruezo et al., 2016). Despite that, those traditional and ancient materials have been displaced, for the last decades, due to the widely adoption of commercial hybrids by farmers, and are at risk of disappearing (Hammer, 2004; Hammer et al., 2003; Lanteri et al., 2003; Votava et al., 2005). In fact, genetic erosion of these materials has only been significantly mitigated by the efforts of several institutions through germplasm collecting expeditions for the past half century, in many centres of diversity (Alonso et al., 2018; DeWitt and Bosland, 1996; Díez et al., 2018; Hammer, 2004).

As a result Spanish genebanks conserve a remarkable diversity for *Capsicum* spp., encompassing the country's most relevant varieties, as well as many foreign and pepper wild varieties (Díez et al., 2018). These collections represent an enormous source of variation for breeding programs, some even helped to develop some of the Spanish most relevant PDOs and PGIs. However, for researchers to exploit the genetic diversity, it is of paramount importance to incentivize exhaustive large-scale characterizations (D'Agostino and Tripodi, 2017). Only then these materials may efficiently contribute to breed new varieties as well as revert variability loss (Egea-Fernández et al., 2018; Fita et al., 2015; Prohens et al., 2017).

Phenomics of *Capsicum* germplasm

Standardized descriptors are crucial regarding germplasm characterization (Figàs et al., 2018b; Gotor et al., 2008; Tripodi and Greco, 2018). However, these descriptors often fail to in-depth characterize traits' range. Other times, descriptors result ambiguous and difficult to evaluate (Brewer et al., 2006; Figàs et al., 2018b). On top of that, morphological characterization is still a tedious task, for the most part performed manually, and highly dependent on the collector's experience, which may have a negative influence on data's accuracy (Brewer et al., 2006). In fact, phenomics has become the bottleneck in crop breeding since the advent of high-throughput genomics, hence the urge to dispose of fast and accurate information on phenotype in order to link those traits to genomic regions to fully exploit available resources (D'Agostino and Tripodi, 2017; Furbank, 2009). On that regard, high-throughput phenomics tools are slowly reducing the gap to genomic studies. These methods have improved by many-fold the phenotyping process by providing fast, accurate and semi-automatic measurements of a large number of traits, otherwise impossible to obtain manually (Brewer et al., 2007; Furbank and Tester, 2011). Hence, one of the most successful phenomics tools is Tomato Analyzer, an extremely versatile digital analysis tool that has transcended its initial goal and is now used for the characterization of a wide range of crops and plant organs (Brewer et al., 2007, 2006; Darrigues et al., 2008; Gonzalo et al., 2009). In addition, Tomato Analyzer utility has been proven many times since it was released. For example, by detecting subtle differences among tomato and eggplant

landraces (Figàs et al., 2014; Hurtado et al., 2013), studying heterosis of agronomic traits of hybrids between eggplant and its wild relatives (Kaushik et al., 2016), studying the morphologic diversity of *Capsicum* species (Tripodi and Greco, 2018), studying heritability of fruit traits of pepper (Naegele et al., 2016), and detecting QTLs associated to fruit traits (Colonna et al., 2019).

Now it has provided important evidence regarding Spanish landraces morphological diversity, for a diverse collection of 109 pepper accessions. Thus, Tomato Analyzer enabled a more powerful intra-varietal and inter-varietal separation, compared to conventional descriptors, particularly for highly related varieties, sharing several fruit, plant and flower traits. These findings are in agreement with those found for tomato and eggplant in previous studies (Figàs et al., 2014; Hurtado et al., 2013). Furthermore, the combination of the most discriminant conventional descriptors with Tomato Analyzer's parameters may enhance pepper fruits characterization while reducing the number of traits to collect. This is of paramount importance towards germplasm typification and conservation, as well as for accession management in seed banks, by providing information of which accessions could represent the most variability and, consequently, help to construct non-redundant core-collections (Lee et al., 2016). The combination of both methods has been implemented in previous reports with satisfactory results (Tripodi and Greco, 2018), however, this is the first time it has been implemented in the study of the *Morrón* peppers of the Spanish centre of diversity. Thus, both methodologies enabled the dissection of the diversity within our collection, confirming that C. *annuum* is an extremely variable species, due to domestication and breeding performed in different regions of the globe with different goals (Bosland and Votava, 2012; DeWitt and Bosland, 1996). Interestingly, fruit traits explained most of the variability of our collection, which is coherent with what was found in other crops, where fruit traits dictacte the varietal types and their use (Costa et al., 2011; Figàs et al., 2014; Hurtado et al., 2013). In pepper, we can observe the same trend and it is fruit shape, colour, and flesh what defines varieties (Bosland and Votava, 2012; Rivera et al., 2016; Tripodi and Greco, 2018). This makes us believe that exploitation of these resources in direct collaboration with farmers and local communities could translate into the development of highly adapted and highly productive varieties that correspond to consumers' demand (Egea-Fernández et al., 2018; Hurtado et al., 2013; Parisi et al., 2017; Zonneveld et al., 2015).

Finally, the development of high-throughput phenotyping tools has a major impact on plant breeding (Yang et al., 2013). However, the high cost and the lack of automatization in the analysis of huge amounts of collected data are relevant issues that scientists still have to address in order to make it as extended as some other tools in the laboratories around the world (White et al., 2012). In the particular case of Tomato Analyzer, the bottleneck is in the post-scanning stage, where there is the need for a technician to confirm or manually correct fruit boundary's delimitation and that distal and proximal

points were correctly assigned (Ramos et al., 2018). This is crucial for an accurate assessment of fruit traits and requires the inspection of every image during the process. Only then, phenomics will be able to provide all of its potential to breeders and be able to respond in an efficient manner to the necessities of crop breeding programs, especially regarding complex traits (D'Agostino and Tripodi, 2017).

Open-pollination effect on *Capsicum* landraces' morphology and diversity

Capsicum annuum was domesticated in Mexico and, as a result, this region is today the primary centre of diversity of that species (Kraft et al., 2014; McLeod et al., 1982; Moscone et al., 2007). Thus, a plethora of varietal types can be found throughout the country's landscape, including some of the world's most known types, such as 'jalapeno' and 'serrano'(Kraft et al., 2010). Centuries of cultivation and selection by generations of farmers combined with a heterogenic landscape gave origin to a remarkable collection of landraces, or *criollos* as they are locally known, which have been perpetuated till this day (Kraft et al., 2010). Landraces encompass a wider genetic diversity, compared to the modern varieties, and are a unique resource with remarkable aptitude to be used as pre-breeding materials or as a diverse population with higher resilience to climate change conditions, fruit of a dynamic evolutive process along with environmental conditions and cultural practices (Aguilar-Meléndez et al., 2009; Kraft et al., 2010; Votava et al., 2005). Notwithstanding, traditional varieties are being replaced by modern varieties, with greater yields and resistances to several stresses (Casals et al., 2011; Lanteri et al., 2003; Rivera et al., 2016). This is particularly true regarding pepper production to be consumed fresh, which limits landraces to the dried market, setting these materials at risk of disappearing (Kraft et al., 2010). Thus, conservation for those genetic resources are of paramount importance for crop breeding and food security (Díez et al., 2018; Hammer, 2004; Hammer et al., 2003).

On that regard, some of the breeding efforts in Mexico have been focused on the improvement and conservation of those traditional varieties, like the 'Ancho' type. In order to do that and to avoid loss of these materials due to genetic erosion, Mexican governmental agencies implemented participatory breeding programs in cooperation with local farmers in order to take advantage of their knowledge to select the best individuals in their fields (Kraft et al., 2010). One of the most important Mexican regions for pepper cultivation is the Aguascalientes state, a small region located in the central highlands of Mexico with a tight link to agriculture and livestock industry (Kraft et al., 2010). Traditionally, farmers cultivate their landraces and at the end of each harvest they selected the open-pollinated seeds to be sown next year. This approach seems to enable a reasonable balance between maintaining a wider genetic diversity while preserving the variety's morphological characteristics (Kraft et al., 2010). Herein we aimed at studying the phenotypic and genotypic diversity within these materials and the open-

pollination effect on fixation of morphological characteristics and on genetic fixation by characterizing two 'Chile Ancho' lines and their progenies, using IPGRI descriptors and SSR molecular markers.

Morphological analysis, given by PCA, showed similar levels of agronomic and morphological uniformity within and between the two open-pollinated families, based on the data from 36 IPGRI descriptors (IPGRI, 1995). Both 'Chile Ancho' families clustered together in the PCA plot, indicating that the standard phenotype is maintained despite the lack of controlled pollination. Hence, two conclusions can be drawn from this results. One, that farmers know these materials and the particular attributes that make them a specific variety. Two, that the open-pollinated method is efficient in terms of reaching enough agronomic uniformity in *criollo* peppers. Kraft et. al (2010) had already reported similar conclusions for this cultivation region, which we now confirm with morphological data (Kraft et al., 2010). In addition, these conclusions were also reported for African pepper landraces (Orobiyi et al., 2017) and for tomato landraces from Catalonia (Casals et al., 2019).

Furthermore, plant and fruit traits explained most of the variation within our collection, as expected, since fruit traits dictacte varietal types and their use for most crops (Costa et al., 2011; Figàs et al., 2014; Hurtado et al., 2013). In pepper, we can observe the same trend (Bosland and Votava, 2012; Rivera et al., 2016; Tripodi and Greco, 2018). In fact, regarding the particular scenario of Aguascalientes, farmers prefer landraces to the modern varieties for dried pepper industry due to their higher dry matter and flavour-related compound contents (Kraft et al., 2010). Another example was given by the work of Orobiyi et al. (2017) where African farmers stated that they select the varieties to cultivate based on fruit aromatic, culinary and technological aptitudes (Orobiyi et al., 2017).

Regarding molecular characterization based on 17 successfully amplified highly polymorphic SSRs, we detected high levels of observed heterozygosity, which seems to be incoherent with the autogamous nature of pepper (Eshbaugh, 1975; Nicolaï et al., 2013; Raw, 2000). However, the DNA pooling of eight plants per accession and the open-pollination conditions may have contributed to this result. Another important factor contributing to this may be the pollen contamination by other varieties, since many farmers usually cultivate more than one traditional variety at a time and in some occasions farmers even cultivate traditional along side with commercial varieties (Casals et al., 2019; Kraft et al., 2010; Orobiyi et al., 2017). In addition, expected heterozygosity was high probably because of the great number of alleles found for our collection, which is coherent with other works in pepper using SSR markers (González-Pérez et al., 2014; Nicolaï et al., 2013; Parisi et al., 2017; Rivera et al., 2016). Other authors have reported high observed heterozygosis values for pepper regarding bigger and more diverse populations than ours, ranging from 1% to 23%, using SNPs (Cheng et al., 2016; Lee et al., 2016). Despite these results, progenies of both parental open-

pollinated lines averaged similar or lower homozygosity levels than their corresponding parental lines, indicating that this cultivation method is efficient at preserving a certain degree of genetic diversity within these materials, which may be of paramount importance for the adaptation to climate change, particularly for poor and low input agricultural systems (Casals et al., 2019; Egea-Fernández et al., 2018; Kraft et al., 2010). Based on molecular data both families are distinguishable, showing that there are genetic differences between the two populations that could be exploited in breeding programs. In addition, within each family there are different levels of genetic fixation despite the phenotypic similarities found by IPGRI descriptors. These results seem to agree with others that origin is one of the most important aspects regarding genetic structure and population diversity (González-Pérez et al., 2014; Taranto et al., 2016).

The study of both morphologic and genetic diversity is an important practice in order to select the most interesting materials to be used in breeding programs (Casals et al., 2019; Rivera et al., 2016; Zonneveld et al., 2015). On that context, the study and improvement of traditional varieties coupled with strategies like participatory breeding could translate into efficient exploitation of that diversity towards the development of improved ecotypes with a higher genetic diversity and adapted to the local conditions while maintaining the variety's main attributes (Casals et al., 2019; Kraft et al., 2010). The exploitation of farmers' knowledge and ability to detect which genotypes may perform better, based on field observations, are of paramount importance for pepper breeding programs, and in combination with researchers knowledge and tools may accelerate breeding programs timelines (Casals et al., 2019). Participatory breeding programs could have a tremendous impact on pepper productions, but have not been implemented as frequently as in other crops (Ceccarelli and Grando, 2019). In the case of 'Chile Ancho' it could help to breed highly adapted varieties to the Aguascalientes conditions by enabling the study of genotype per environment interaction and by selecting those traits that have a higher acceptance by the consumers, making these materials more competitive with modern ones (Kraft et al., 2010). These findings are crucial towards genetic resources conservation, to climate change mitigation and to help to boost food production without increasing the toll on the environment.

Genomics of *Capsicum* germplasm

As opposed to phenomics, where improvements have been slow, genomics has been a thriving subject, driven by technology that has come a long way since its origins (D'Agostino and Tripodi, 2017). Bear in mind that the first DNA sequences, 1970's, were limited to a few model organisms and the costs were prohibitive for almost every laboratory in the world (Sanger et al., 1977). Since then, methodologies have improved and became cheaper with time, thus, sequencing an entire human genome, for example, drop from $300 million, in the year 2000, to $14 million around 2006, and finally to less

than $1000, today (NIH, 2019). More recently, high-throughput sequencing technologies have cut sequencing costs by several-fold, enabling for researchers to have access to high quality reference genomes for most of the relevant crops in the world (Elshire et al., 2011; Genome 10K Community of Scientists, 2009; Poland and Rife, 2012). Having high-quality reference genome is a powerful resource for plant breeding that enables the study of the genomic structure, of evolutionary processes and the understanding of genomic elements function (Qin et al., 2014). Ultimately, it enables precise gene and gene function dissection (Hulse-Kemp et al., 2018; Kim et al., 2017). In *Capsicum*, availability of several reference genomes in combination with next generation sequencing technologies, like genotyping-by-sequencing, has successfully been applied to generate thousands of genome-wide highly informative SNPs. These are extremely helpful tools, which can be then applied for downstream analysis in order to study germplasm diversity, population structure and genomic selective sweeps, as well as, genome-wide association studies for the detection of trait-associated QTLs (Ahn et al., 2018; Cheng et al., 2016; Nimmakayala et al., 2016a; Taitano et al., 2018; Taranto et al., 2016).

Herein we used high-throughput genotyping-by-sequencing to study a collection of *Capsicum* spp., encompassing a comprehensive collection of Spanish heirlooms along with several accessions from distinct centres of diversity and species, in order to shed light into the Spanish landraces phylogenetic relationships and to evaluate their genetic diversity and population structure. On that matter, accessions DNA samples were successfully digested with *Ape*KI restriction enzyme and libraries prepared as described by Elshire et al. (2011). Illumina HiSeq2500 (Illumina, Inc., San Diego, CA, USA) single-end technology was used for library sequencing, generating 6,766,231 high quality unique read tags (Elshire et al., 2011). Finally, SNP calling was successfully performed against reference genome CM334 version 1.5 (Kim et al., 2014), yielding 4083 genome-wide highly informative SNPs which were used for genetic diversity and phylogeny analysis (Glaubitz et al., 2014).

Based on 4083 GBS-generated SNPs, phylogenetic studies confirmed the close relationship among Spanish landraces, and other European accessions, with materials from the Mexican centre of diversity. This findings confirmed that Spanish materials resulted from an important influx of germplasm from Mexico, during the Discovery Era (Andrews, 1995; Nuez et al., 2003). Another important result is the intermediate position of Spanish materials, which located in between the Mexican and the rest of European materials, although far closer to the European than to the Mexican accessions. This is consistent with the available knowledge about the diffusion of *Capsicum* materials throughout Central Europe after their introduction in Spain (Andrews, 1995; Eshbaugh, 1983; Nuez et al., 2003). In addition, European accessions represent a narrower genetic diversity compared to the related materials from Mexico, which may be due to a founder effect, since they descend from a reduced set of individuals brought from Mexico

(Andrews, 1995). Another factor contributing to the narrow diversity among European accessions could be the widely adoption of the same modern varieties, encompassing the same genetic background, could be altering genetic structure by pollen contamination (Ibiza et al., 2012).

Furthermore, phylogenetic analysis provided important insights into the origin of the *annuum* complex and its relation with *C. baccatum* and *C. annuum* var. *glabriusculum*. Thus, *C. annuum*, *C. baccatum* and *C. chinense* accessions were successfully separated whereas *C. frutescens* accessions clustered together with *C. chinense*, and *C. annuum var. glabriusculum* accessions were spread into two distinct genetic pools. The separation of some 'Chiltepins' into one pool, closely related to *C. annuum*, and the rest into another, closely related to *C. chinense-frutescens* cluster, indicates a possible common ancestry, as proposed by several authors (Hayano-Kanashiro et al., 2016; Moscone et al., 2007; Qin et al., 2014). Finally, *C. baccatum* situated in between *C. chinense-frutescens* cluster and the *C. annuum* group, although closer to the former. According to McLeod's et al. (1982) theory, *C. baccatum* originated in the Bolivian dry lowlands, while *C. annuum* complex species, *C. chinense* and *C. frutescens*, originated outside the Bolivian "nuclear area" but in relatively close regions, in the Amazon basin (McLeod et al., 1982; Moscone et al., 2007; Scaldaferro et al., 2018). *Capsicum annuum*, on the other hand, was domesticated significantly farther north, in Mexico, where has its centre of diversity today (McLeod et al., 1982; Moscone et al., 2007). Hence the genetic proximity among these taxa in our results and not the expected disposition where *C. frutescens* (González-Pérez et al., 2014; Nicolaï et al., 2013) or *C. chinense* (Lee et al., 2016) locate closer to *C. annuum*. Interestingly, according to the three methods used herein to assess the genetic relations and population structure, *C. chinense* and *C. frutescens* were inserted in the same cluster, showing a close relation between these materials, possibily indicating that these should be considered a single species. In fact, this debate has always been present when it comes to *Capsicum* taxonomy and many authors believe these two taxa should be merged into one (McLeod et al., 1979b; Walsh and Hoot, 2001). However, in a different study, authors reliably distinguished the two species based on morphological descriptors (calyx constriction and flower position), RAPD molecular markers, and their hybrids fertility, providing evidence that these are two separate taxa (Baral and Bosland, 2004). Unfortunatelly, the reduced number of individuals included in our study does not allow for strong conclusions.

Finally, the study of genomic landscape based on Tajima's D statistic revealed several purified genomic regions associated to domestication and to positive selection within the *C. annuum* materials. Thus, high Tajima's D values for a region spanning 7.5 Mb in chromosome 1 has been reported as having QTLs implied in fruit weight, length and diameter, and pedicel length (Ahn et al., 2018; Barchi et al., 2009; Hill et al., 2017). Likewise, chromosome 5 showed a purified region spanning 6 Mb and possibly linked

to fruit diameter (Ahn et al., 2018; Hill et al., 2017). Finally, high Tajima's D values were found for a 1 Mb region in chromosome 6 linked to the control of several fruit traits such as weight, diameter, pericarp thickness (Chaim et al., 2001; Hill et al., 2017; Rao et al., 2003; Yarnes et al., 2013). As expected, clusters comprising mostly *C. chinense*, *C. frutescens*, *C. baccatum*, and 'chiltepins' showed a balanced distribution of Tajima's D values, coherent with low or inexistent positive selection and within the normal values for traditional materials with little to none breeding selection and often cultivated in isolated areas where genetic flow is extremelly complicated (Albrecht et al., 2012; Hayano-Kanashiro et al., 2016; Ibiza et al., 2012; Moses and Umaharan, 2012). Our results are in agreement to those of other works for both *C. annuum* and *C. baccatum* for which researchers reported purified regions (Nimmakayala et al., 2016b; Taitano et al., 2018).

These findings provide relevant information of the origin and relationships of Spanish landraces and for future association mapping studies in pepper. In addition, our results emphasise the importance of fruit traits and region of origin as major factors controlling genetic structure and genetic diversity among our collection (Aguilar-Meléndez et al., 2009; Ibiza et al., 2012; Naegele et al., 2016). Ultimately, we provide important tools regarding pepper breeding, and validate the applicability of genotyping-by-sequencing in pepper genetic studies.

Fruit quality in *Capsicum* germplasm

Improved fruit quality is a major goal for pepper breeding programs. However, fruit internal quality was relegated to a second plan while breeding programs focused their efforts on yield, resistance and external quality, resulting in loss of sensorial attributes (Casals et al., 2011; Egea-Fernández et al., 2018; Parisi et al., 2017). Recently, interest for the "taste of the past" is contributing to the re-introduction of landraces due to consumer perception of these materials as a better source of nutrients and richer in flavour compared to modern varieties (Brugarolas et al., 2009; Hurtado et al., 2013; Rivera et al., 2016). As a result, breeders are now focusing more efforts into improving internal content (Egea-Fernández et al., 2018).

There are several works regarding the nutritional content of pepper pods. However, those were not equally distributed, regarding species or compound of interest. Hence, *C. annuum* has concentrated most of research efforts (Ribes-Moya et al., 2018; Rodríguez-Burruezo et al., 2010; Wahyuni et al., 2011), whereas *C. baccatum* only recently started to get attention, despite its known relevancy in other pepper breeding aspects, such as resistance to biotic stress and genetic diversity source (Ahn et al., 2018; Rodríguez-Burruezo et al., 2009; Yoon et al., 2006). Likewise, phenols, volatiles and carotenoids have concentrated most of the efforts put into *Capsicum* fruits internal

content characterization (Olguín-Rojas et al., 2019; Parisi et al., 2017; Ribes-Moya et al., 2018; Wahyuni et al., 2011; Zonneveld et al., 2015).

Capsicum fruits are a good source of vitamins (Sarpras et al., 2019), being pepper's antioxidant activity mostly due to the vitamin C and it is not unusually to find materials that are able to provide the daily recommended dose of vitamin C with less than 100 g of fresh weight (Ribes-Moya et al., 2018; Rodríguez-Burruezo et al., 2013a, 2011). As a result, breeding for high content in vitamin C has been a secondary goal although it is still measured as a relevant indicator of nutritional quality (Rodríguez-Burruezo and Nuez, 2006). Furthermore, pepper is also a good source of macro and microminerals (Pérez-López et al., 2007b; Rubio et al., 2002; Sarpras et al., 2019). However, contrarily to other health-promoting compounds, mineral content has only been studied for a reduced number of varieties and *C. annuum* concentrated most of those efforts (Pérez-López et al., 2007b; Rubio et al., 2002).

Improving fruits for their content of health-promoting compounds, such as vitamin C and minerals, would have a positive impact on human health (Yahia et al., 2019). Despite that, improving fruit internal content is going to be tremendously difficult, because modern varieties have evolved into a diversity bottleneck that completely altered its metabolomics and, because of the polymorphic control of quality traits, significant advances in this area are slow (Casals et al., 2011; Egea-Fernández et al., 2018). In order to accomplish that goal, researchers need to search for new sources of variation for those traits (Parisi et al., 2017; Ribes-Moya et al., 2018; Rodríguez-Burruezo et al., 2009). On that regard, *C. baccatum* is known as a remarkable genetic pool for several traits of interest and is an asset of unexplored variability and richness in bioactive compounds (Rodríguez-Burruezo et al., 2009; Scaldaferro et al., 2018; Yoon et al., 2006; Zonneveld et al., 2015). The introduction of this species in breeding programs could be a huge step towards the improvement of pepper internal quality. Plus, with the increase of South American immigrant population in Europe and the increasing interest of the Europeans in ethnic food, *C. baccatum* varieties are becoming increasingly demanded outside their natural region of cultivation and the opportunity to improve this species adaptation outside de Andean region has not been fully exploited yet (Rodríguez-Burruezo et al., 2009; Ugás and Mendonza, 2012). With that in mind, we aimed at studying the protein (PRT), ascorbic acid (AA) and mineral profiles, as well as their adaptation to Mediterranean cultivation conditions, of a set of *C. baccatum* accessions as first step towards the selection of individuals with most potential to be exploited in breeding programs for improved fruit quality under Mediterranean conditions.

We observed, herein, a great variability regarding quality traits, corroborating that *C. baccatum* is a diverse species (DeWitt and Bosland, 1996; Zonneveld et al., 2015). Plus, we observed a good adaptation of these materials to the Mediterranean conditions under

both greenhouse and open-field conditions, opening the possibility of exploiting these materials outside their region of origin. This is coherent with the findings of previous works with this species under these conditions (Rodríguez-Burruezo et al., 2009). *Capsicum baccatum* constitutes an important unexploited gene pool that has been grown in the Andean regions for thousands of years (Ibiza et al., 2012; Rodríguez-Burruezo et al., 2009; Scaldaferro et al., 2018). Now, the possibility to cultivate it outside that environment makes it easier for resaerchers to take advantage of its potential. *Capsicum annuum*, on the other hand, has been cultivated in Spain for 500 years, since its introduction from Mexico, therefore, adaptation to the Mediterranean conditions is not an issue (Andrews, 1995; Nuez et al., 2003). In addition, *C. baccatum* has not been subjected to intensive breeding efforts, as opposed to *C. annuum*, and is still cultivated, in most parts, as a traditional variety or as semi-wild shrubs with small fruits, and often in isolated areas where the genetic flow is low (Ibiza et al., 2012; Scaldaferro et al., 2018). Plus, *C. baccatum* can only be naturally crossed with itself or other species from its complex (Ince et al., 2010; Walsh and Hoot, 2001). This may explain the differences of fruit size observed for these two species, since increased fruit size is a result of the domestication process and selective pressure towards that phenotype (Pickersgill, 1997, 1971).

According to our results, fruit internal content is highly dependent on genotype, environment and genotype × environment interaction. This result is in agreement to other reports' claims, not only for pepper but for several other crops and vegetables (De Pascale et al., 2016; Figàs et al., 2018a; Guijarro-Real et al., 2019; Ribes-Moya et al., 2018; Rodríguez-Burruezo et al., 2013a). The study of how these effects change the health-promoting compounds accumulation in vegetables is of paramount importance in order for breeders to select the best candidates to be introduced in breeding programs for a specific cultivation system or environment (De Pascale et al., 2016). This is of special importance in a scenario where all resources must be efficiently used, such as the one we are in the advent of entering, with a substantial population increase and the effects of climate change (Mogollón et al., 2018; Raza et al., 2019).

Ascorbic acid showed a positive increment when plants were cultivated under OF condition, up to 2-fold in some cases, under OF conditions and for *C. annuum* accessions. This is a well documented behaviour in pepper (Ribes-Moya et al., 2018; Rodríguez-Burruezo et al., 2009) and researchers have linked the increment of ascorbic acid to the role it plays in the plant's defense mechanism. Hence, like many other bioactive compounds, its concentrations increases as protection measure against biotic and abiotic stresses, like UV radiation and heat-stress (Smirnoff and Wheeler, 2000). Our values are similar to the ones found for *C. baccatum* and slightly higher for *C. annuum* in previous works (Pérez-López et al., 2007a, 2007b; Ribes-Moya et al., 2018; Rodríguez-Burruezo et al., 2009).

Likewise, protein and mineral content where significantly affected by cultivation, environment and genotype. In general, OF conditions provided higher concentrations of protein, Mg, Ca and Zn. Whereas P and K were, on average, higher under greenhouse conditions. We observed a reduction of fruit average weight under open-field conditions, compared to greenhouse grown plants, which could be indicating that under these conditions fruit growth is restricted to a certain level. Since the irrigation solution was equal in both conditions, differences among mineral and protein concentrations may be caused by a reduction of hidration levels and other abiotic factors which reduced cell division, resulting in an increment of mineral concentrations. In addition, *C. annuum* accessions showed higher concentrations for most of the mentioned traits. In fact, *C. baccatum* accessions showed only a higher concentration than *C. annuum* for K, under both cultivation environments, and Mg, under OF conditions. Furthermore, our findings regarding K, Mg, Ca, Fe, and Zn are in agreement with other works (Pérez-López et al., 2007b; Sarpras et al., 2019). P levels, on the other hand, were higher than in other works with pepper (Sarpras et al., 2019). Regarding these results, *C. annuum* accessions high adaptation to the Mediterranean conditions, as result of several years of selection and breeding by our research group, may be enabling a better performance, compared to *C. baccatum*, regarding mineral uptake and accumulation. Furthermore, *C. baccatum* is mostly cultivated in the high mountainous areas of Bolivia and Peru and its cultivation outside those areas is within the first generations and, therefore, breeding for productivity is still needed (Ibiza et al., 2012; Rodríguez-Burruezo et al., 2009).

Capsicum baccatum accessions, showed that a single portion of 100 g of fresh fruit can provide on average 50% (under greenhouse conditions) up to 90% (under open-field conditions) of the recommended dietary day allowance for ascorbic acid, and between a 5 and 60% of the recommended dietary day allowance for for the minerals studied here. These results provide an optimistic insight into the possibility of improving *C. baccatum* regarding yield and bioactive compounds content in the near future under Mediterranean conditions. However, there is still room for improvement, particularly regarding this species estability, yield and internal content in both bioactive compounds and minerals concentration. In fact, improving organoleptic and nutritional quality of a crop does not only provide benefits to human health; in addition to that, improved varieties would have a higher resilience to biotic and abiotic stresses, since several of these compounds have a protective effect (Gould, 2004; Landi and Tattini, 2015; Luna-Ruiz et al., 2018; Pourcel et al., 2007; Smirnoff and Wheeler, 2000) and, at the same time, we would be developing added-value varieties with a good acceptance by consumers (Casals et al., 2011; Egea-Fernández et al., 2018; Parisi et al., 2017; Rivera et al., 2016; Rodríguez-Burruezo et al., 2009; Rubio et al., 2002).

Hence, the criation of active breeding programs towards improving pepper pods internal mineral and vitamin content would be very productive, while providind insights into the nutritional content controlling mechanism. In the advent of NGS technologies, the study

of complex traits, such as fruit quality, has become considerably easier and the evaluation of the available germplasm and the selection of the best indiviuals could provide vital insights into the genetic mechanisms controlling these traits, opening the way to the introgression of those alleles into new cultivars (D'Agostino and Tripodi, 2017; Rodríguez-Burruezo et al., 2005; Yarnes et al., 2013).

Capsicum adaptation to low phosphorus input

Addition of P-enriched fertilizers is usually the adopted strategy to replenish P levels after each harvest (Cordell et al., 2009; Mogollón et al., 2018). However, this practice is neither environmental or socio-economically sustainable. In fact, most of the added fertilizer is lost through lixiviation and soil erosion (Lynch, 2007; Tilman et al., 2002; Vance et al., 2003), resulting in contamination of water bodies (Fernández and Selma, 1998; Kauranne and Kemppainen, 2016). Adding to that, the severe carbon and other contaminants emissions resulting from this mineral's mining have a negative footprint on the environment (Cordell et al., 2009; Tilman et al., 2002). On top of that, rock-phosphate is a finite resource that could be depleted before the end of this century (Cordell et al., 2009; Schnug and Haneklaus, 2016b; Vance et al., 2003). Therefore, P is becoming an extremely expensive resource and, without any measures to use it more efficiently, access to chemical P-enriched fertilizers will become limited to those coutries who can afford it, affecting mostly poor and developing nations (Cordell et al., 2009; Schnug and Haneklaus, 2016b; Vance et al., 2003).

Hence, improving crops ability to uptake and efficiently use P would produce a significant impact on the reduction of rock-P mining and on agriculture fertilizer applications, while helping to boost poor and developing nations yields (Schnug and Haneklaus, 2016b). However, in order to accomplish these goals, researchers must understand the mechanisms underlying the response to low phosphorus conditions. Regarding that, several authors have linked root architecture to enhanced mineral acquisition. Those studies indicate that root adaptations, such as increment of number of lateral roots, root hairs and cluster roots, change of root architecture to a topsoil foraging system, increment of organic acids production and phosphatases, root P transporters enhanced expression, and root cellular structure alteration, are found in a vast number of species and are correlated to a greater performance under low P conditions (Niu et al., 2013). Furthermore, several authors have reported genomic regions controlling some of those adaptations for several crops, indicating that these are transversal to any crop, including pepper (Hammond et al., 2009; Li et al., 2009; Lynch and Brown, 2001; Zhu et al., 2005). Despite that, pepper fundamentals regarding its adaptation to P-stress conditions are still unravelled. In order to close this gap within crop and particularly in pepper breeding, we established as main goal the characterization of the main root adaptations of *Capsicum* spp. to low P conditions as a

first step towards the identification of elite individuals for future pepper breeding programs for improved P uptake and use efficiency.

Our findings indicated a significant decrease in both P concentration and accumulation in the plant's root, shoot and fruit tissues, when plants were cultivated under low P conditions. We observed that organs are able to mobilise internal P when in stress to where it is most needed, in order to keep the plant alive. Hence, roots are able to completely alter their P needs, in order to favour above ground biomass. In order to do this, roots have been reported to change their structure by stimulating the growth of thinner roots and by replacing its cells with aerenchyma or higher porosity, in order to decrease metabolic expenses and P requirements of the root system, while maintaining their foraging ability (Fan et al., 2003; Fernandez and Rubio, 2015). Furthermore, the response among accessions was remarkably diverse, regarding P level loss and regarding P allocation under stress treatment, indicating the possibility of selecting materials with higher capability of mobilising fruit P to other organs and, consequently, with less need for fertilizer applications.

In addition, stress treatment caused a significant dry weight loss in all of plants' organs, as it would be expected, particularly noted for the aerial part of the plant. However, the loss was not equal to all genotypes, thus, several strategies can be perceived through the analysis of how the loss of biomass affected each genotype. For example, some genotypes promoted root growth instead of aerial in order to enhance its foraging capability, whereas others adopted a more conservative strategy and reduced the roots total biomass and consequently reduced their need for P. These behaviours have already been reported in other works and sometimes within the same species, differential responses are observed (Fernandez and Rubio, 2015; Li et al., 2009). The availability of response variation is of paramount importance for researchers to select the individuals whose response fits most with their goals. Furthermore, loss of RW, as seen in our experiment, may indicate an adaptative response by the genotype in order to enhance its P uptake from the soil by increasing the formation of thinner roots with increased porosity and aerenchyma in their structure (Fan et al., 2003), as well as the formation of root hairs instead of lateral roots (Bates and Lynch, 2001; Fernandez and Rubio, 2015; Hammond et al., 2009). Other adapative strategies observed in our trials and in previous works are the increase of the average length of the radicular system, particularly the length dedicated to root hairs, instead of primary or lateral roots, as well as the increase of distance units of root per weight of the root (Bates and Lynch, 2001; Fernandez and Rubio, 2015; Hammond et al., 2009; López-Bucio et al., 2003). All these adaptations or strategies aim at enhancing P acquisition without increasing metabolic costs by exploiting a bigger fraction of the soil and by enhancing P uptake and use efficiency. The understanding of these adaptations is of paramount importance to the dissection of the regions controlling them. Methodologies have improved by many-fold the execution time of characterization of entire root systems (Paez-Garcia et al., 2015; Pereira-Dias et

al., 2015a). Despite that, the study of root and its architecture is still an arduous task and remarkably prone to errors and misinterpretation, especially when studies are performed under true soil conditions (Paez-Garcia et al., 2015; Pereira-Dias et al., 2015a).

Improving pepper P uptake and use efficiency would have a significant impact in reducing the need for chemical fertilizers. With that in mind, an exhaustive germplasm screening should be incentivized in order to promote the discovery of candidate materials for improved root architecture and enhanced P uptake and use efficiency. Likewise, the ability to link phenotype to genotype is of paramount importance in order to introgress genomic regions controlling traits of interest into other materials (Prohens et al., 2017). Notwithstanding, characterization of resistance to abiotic stresses is complex, although, major findings have been reported for pepper in the recent years, such as heat (Guo et al., 2016, 2014; Li et al., 2015), drought (Hong and Kim, 2005; Huang et al., 2019; Sahitya et al., 2019; Shivakumara et al., 2017), cold (Hwang et al., 2005; Li et al., 2016), and salt (Bojórquez-Quintal et al., 2014; Jing et al., 2016). Despite that, we are still far from fully understand the mechanisms involved in response to abiotic stress and, therefore, far from being able to introgress those traits into cultivars (Russo, 2012). Next-generation sequencing tools and genome-wide association studies have already demonstrated potential identifying genomic regions controlling traits of interest and providing thousands of molecular markers linked to said traits (Ahn et al., 2018; Celik et al., 2017; Guo et al., 2016; Nimmakayala et al., 2016a; Pascual et al., 2015). The use of high-throughput phenotyping in combination with high-throughput genotyping methods enables large scale characterization of germplasm collections and, therefore, may result in an accurate understanding of the mechanisms underlying the response to P depravation (Chhapekar et al., 2018; He et al., 2014; Paez-Garcia et al., 2015).

General conclusions |

| The in-depth characterization of a highly representative collection of the most relevant heirlooms and landraces from the Spanish centre of diversity enabled the detection of a remarkable intra and inter-varietal variation for several agro-morphological traits. These findings will be very useful for future breeding programs and future association studies.

| The application of high-throughput phenomics tools was a crucial complement to conventional descriptors, providing fast and comprehensive insights into pepper fruit shape at a level of detail not possible manually, and enabled the detection of a reduced group of highly discriminant traits, which accurately separates closely related materials. This has special implications in both germplasm management and in the reduction of phenotyping efforts.

| The combination of both farmer's individual selection of the best individuals and the open-pollination cultivation system proved to be an effective and low-cost practice to improve chile *criollo* lines, while maintaining the desired morphotype of the variety and a certain degree of genetic diversity.

| Based on 4083 GBS-generated higly informative SNPs, phylogeny studies confirmed that Spanish landraces arouse from Mexican materials and that *C. annuum* var. *glabriusculum* is the likely ancestor of the *annuum* complex, revealing as well an important reduction of genetic diversity within Spanish and other European materials, compared to the Mexican centre of diversity accessions, likely due to founder effect. In addition, fruit traits and region of origin are the most important factors controlling the genetic structure underlying our germplasm collection.

| The characterization of the internal content showed a wide variation for bioactive compounds and mineral content for *Capsicum*, and particularly within *C. baccatum*, revealing this species as a valuable source of variation to improve fruit quality.

| Our findings corroborate that growing environment is a major factor affecting nutritional content of pepper pods and that for the collection considered herein; open-field conditions would have a greater impact improving accessions fruit internal quality. These findings are important to successfully improving *C. baccatum* health-promoting compounds profile.

| For the first time, main pepper adaptations to low input phosphorus conditions were studied, linking root traits to parameters of phosphorus acquisition and use efficiency. In general, stress treatment stimulated root growth and the formation of thinner roots. The characterization of the different genotypes' behaviour under low input conditions detected a considerable amount of variability, opening the possibility to selection towards several traits and behaviours and combining them into a single variety. These findings are of paramount importance for the development of more resilient varieties and the reduction of the need for chemical fertilizers.

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