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Fenotipado radicular para la resistencia a la sequía en Solanum spp.

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AUTORA: ANASTASIA AINHOA ATUCHA ZAMKOVA

TUTORA: ANA MARÍA FITA FERNÁNDEZ

COTUTOR: JAIME PROHENS TOMÁS

COTUTOR EXTERNO COLABORADOR: MARIA DE LA O PLAZAS ÁVILA

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RESUMEN

Fenotipado radicular para la resistencia a la sequía en Solanum spp.

La sequía es un gran problema para la agricultura, ya que destruye o merma considerablemente las cosechas. Por eso la mejora genética de las plantas para la tolerancia a sequía es una necesidad acuciante. Para mejorar las plantas genéticamente, es necesario tener herramientas de fenotipado adecuadas para poder identificar las plantas con las características deseadas. La berenjena cultivada es una planta que es bastante tolerante a la sequía, y posee una gran variedad de especies relacionadas que podrían ser interesantes para introgresar más genes de tolerancia al estrés hídrico. Desarrollar un buen protocolo para el fenotipado de las raíces podría ayudar a seleccionar plantas resistentes a sequía.

A la hora de fenotipar ha sido un problema el que tanto la berenjena como las especies silvestres relacionadas presentan problemas de germinación o de latencia. Por este motivo los objetivos de este trabajo fueron: a) desarrollar un protocolo de germinación rápida para la berenjena y sus especies relacionadas y b) desarrollar un protocolo de evaluación de los sistemas radiculares en planta joven *in vitro* en condiciones de estrés y c) estudiar los sistemas radiculares de la berenjena y otras especies relacionadas en condiciones de estrés.

Para obtener un protocolo optimizado de germinación se planteó un diseño ortogonal donde se probaron combinaciones de diferentes tratamientos (sumergir las semillas en agua, luz, calor, oscuridad, aplicación de GA_3 o KNO_3) que en la literatura se describen como positivos para la germinación. Se utilizaron tanto semillas jóvenes con latencia (menos de un año tras la recolección del fruto) y viejas (sin latencia). Los resultados mostraron los valores positivos para la germinación de semillas jóvenes de sumergir las semillas en agua, aplicar KNO_3 y dejarlas a la luz. Se observó también que la lejía a la concentración utilizada (al 30%) es muy dañina para las semillas. Por otro lado se observó que las semillas viejas y jóvenes respondieron de forma diferente a los tratamientos, probablemente debido a la presencia de factores de latencia en las últimas.

La técnica empleada para el fenotipado de las raíces fue crecer las plantas en placas mediante cultivo *in vitro*. Esta técnica permite estudiar la arquitectura de las raíces en la planta joven y aplicar distintos tratamientos. En nuestro caso utilizamos dos tratamientos: uno control y otro con polietilenglicol que simulaba el estrés hídrico. Aunque por problemas en la germinación no pudimos evaluar todos los genotipos planteados en el experimento, los resultados mostraron que existen diferencias en la arquitectura radicular de la berenjena y sus especies relacionadas, dando lugar a respuestas adaptativas de la raíz al estrés hídrico.

ABSTRACT
Root phenotyping for drought resistance in Solanum spp.

Drought is a very important problem for modern crops, because it destroys or significantly reduces them, even when modern irrigation technology is used. That's why genetic breeding of plants for tolerance to drought is vital. But in order to do the breeding, it is crucial to have the right phenotyping tools that would help to identify the plants with the desired characteristic. The cultivated eggplant is quite tolerant to drought, and has a great variety of related species that could be used to introgress genes for water stress tolerance. Developing a good protocol for the phenotyping of the roots could help in the backcrossing program.

While phenotuping the roots, the problem of the poor germination of the seeds of wild species and the eggplant hybrids was encountered. That is why the objectives of this wor include a) developing a protocol for the rapid germination of the eggplant and its related specied; b) developing a protocol to evaluate the root

architecture of the young plant in vitro in stress conditions; and c) studying the root system of the eggplant and related species in stress conditions.

To develop a protocol for seed germination, an orthogonal array design was done, and it included eight combinations of the treatments (imbibition with water, light, heat, applying GA_3 and KNO_3). Young, dormant seeds (less than a year old) and old, non dormant seeds were used. The treatments that were proved to have a positive effect on the germination of young seeds were submerging them with water and applying KNO3, as well as leaving them under the light. Bleach, which is used for the disinfection of the seeds for in vitro, was found to be very harmful for the seeds at the concentration that was used (30%). Young and old seeds responded differently to the treatment, because of the dormancy of the young seeds.

The technique used to phenotype the roots was growing them on plates in *in vitro* culture. This technique lets us study the architecture of the roots of young plants, applying different treatments. Two treatments were used: a control one and a water stress one, simulated by polyethylene glycol. And, although due to problems in germination not all the genotypes could not be evaluated, results showed the differences in the root architecture of the eggplants and the related species, proving the existence of adaptation to water stress.

PALABRAS CLAVE: Berenjena, raíces, germinación, polietilenglicol, *in vitro*

KEY WORDS: eggplant, roots, germination, polytethylene glycol, *in vitro*

ALUMNA: Anastasia Ainhoa Atucha Zamkova

Valencia, a 7 de Julio del 2015

TUTOR ACADÉMICO: Prof. Dña. Ana María Fita Fernández

COTUTOR: Jaime Prohens Tomás

COTUTOR COLABORADOR: María de la O Plazas Ávila

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ABBREVIATIONS

C (1-8): Combination (1-8)

CIMMYT: International Maize and Wheat Improvement Center

MABC: Marker-assisted backcrossing

MAS: marker-assisted selection

NIL: Nearly introgressed lines

PEG: Polyethylene glycol

1. INTRODUCTION

1. 1. Fighting the drought

1. 1. 1. Drought and its economic importance

At a time in which the world population is already surpassing 7 billion people (United States Census Bureau, 2015), an adequate supply of food is crucial in order to feed it. Maintaining the food supply adequate may be hard, especially when the natural conditions complicate food production. One of the most important natural (and therefore uncontrollable) factors that affect the size of the food supply is the climate.

Usually climate is predictable, giving predictable precipitations at known times, like in the regions with mild, Atlantic weather, controlled by the Gulf stream. But sometimes unpredictable events happen, or human do not have the tools to react appropriately to the extreme weather conditions. One of the climate phenomenas that is hard to control is drought. Severe drought has, throughout history, reduced the available supply of food, causing starvation and death, like the Great Famine in India that lasted from 1876 to 1878, that killed around 30 million people, or the Sahel drought, that lasted from 1970 to 1980, that led to 600,000 deaths (Heffernan, 2013). These famines affect the poorest people the most (FAO, 2015). Drought also may precede civil unrest, like in the case of Syria. Even when drought does not lead to death or war, farmers in impoverished countries are forced to reduce the quantity of livestock they have, even sometimes being forced to sell the oxen and donkeys, necessary for plowing the land (FAO, 2015). It can also lead to severe crop losses and economic damages, increasing prices of crops and making them more expensive for poor farmers.

This makes it necessary to study the drought, finding patterns and classifying it accordingly, in order to be able to have a specific response to each part of the problem. According to Heffernan, (2013) the term drought can refer to three different aspects: meteorological drought, which occurs when precipitations diminish, hydrological drought, when water in reservoirs, aquifers, etc. dries out, and soil-moisture drought, which is caused by evapotranspiration that makes the soil go dry (this is the one that affects plants, but is aggravated by the two previous ones, among other factors, such as soil drainage).

Climate change affects the frequency and quantity of droughts, affecting global temperatures and global climatic phenomenae, making meteorological droughts more frequent and severe. For example, it has been predicted in mathematic models that climate change will lead to more severe El Niño-La Niña oscillations (Timmerman, 1999). The two climate phenomenae have already caused severe droughts in some parts of the world and heavy rainfalls in other parts, affecting human civilizations during all our history. A particularly severe el Niño caused the demise of the Moche, a pre-Colombian civilization from Peru (Dillehay et al., 2004). If the droughts caused by el Niño are very severe, this could affect the future of civilization, diminishing the amount of food.

However, sometimes drought is not just an occasional catastrophe, but a yearly event, like in arid regions. Semi-arid regions do have precipitations, but often they are not enough to sustain water hungry crops without irrigation. And a lot of the world's fertile lands (California, Texas,

Spain and the Mediterranean regions) are semi-arid (Figure 1.1), making irrigation necessary for agriculture.
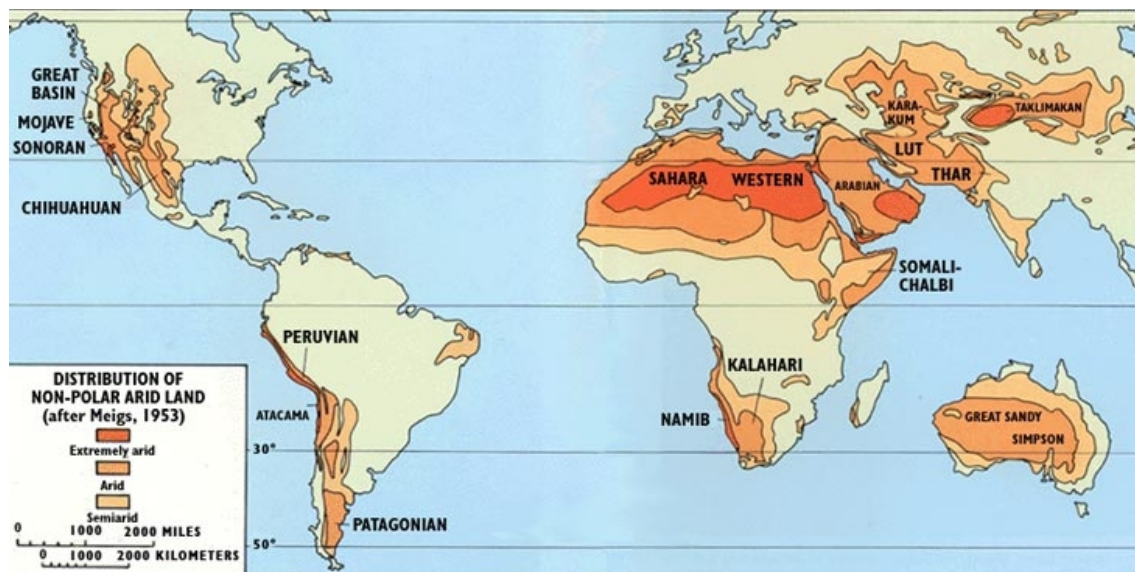

Figure 1.1. A map of the world's semi-arid and arid regions (USGS, 2015).

The problems caused by meteorological drought can be palliated by irrigation. However, the careless waste of water in great part of our modern agricultural systems is increasing the hydrological drought, already accentuated by meteorological drought. In California, where the water for irrigation comes from the snow packs in Sierra Nevada, the warming of the climate and the decreased amount of snow has severely reduce water reserves.

Water, and its use, has caused conflicts during all history, and a lot of conflicts in the XXth century, like the conflict between Syria and Israel around the Jordan river, in 1965-1966, and the long conflict over the Nile waters between Egypt and Ethiopia. Most recently, the problems caused by La Niña, a global climate phenomenon, has caused severe drought in California, that forced them to limit water for home use by 25% (the meteorological drought caused hydrological drought, reducing the quantity of water), or the reduction by half of the crops in Ethiopia, Somalia and Kenya.

1.1.2 Technologies used against the drought

Responses to drought should involve all members of society, from scientists to farmers and policymakers. Policies can help to decrease social damage and to adapt to meteorological conditions better. Crops that require a lot of water could be substituted by other, less water hungry crops, if it is known in advance that the weather will be dry.

In some parts of the world drought has begun to be chronicle due to climate change, and water reservoirs have started to dwindle. This means that in the future, there will be much less freshwater available for agriculture. Currently, 70% of the world freshwater use is for agriculture (Bourzac, 2013). Part of the problem can be solved by using more efficient systems of irrigation. The efficiency (percentage of water used for transpiration and growth) of these systems varies widely; for example, farrow irrigation is 65% efficient, sprinklers are 75% efficient, and micro-irrigation is 90% efficient. But the most efficient system, micro-irrigation,

also called drip irrigation, is also the most expensive one, and the capital costs required make it useful for expensive crops like fruits and vegetables, but too expensive for commodity crops like soy and maize.

Another problem is that most of the water, even if used by the plant, is lost during transpiration. In a study by Segal et al. (2006), done in sunflowers, it was shown that continuous irrigation improved water uptake efficiency and yield, especially in young plants. Other efficient irrigation technologies, like the one used by O'Shaughnessy et al.., 2012, where sorghum was watered only when it was suffering from water stress, also help to save water. A novel idea that could help to save water by retaining moisture in soil and by helping plants to absorb water is to add mychorrizal fungi to the soil and roots (East, 2013).

In some parts of the world, though, the levels of available freshwater are so low that even with the most efficient irrigation systems and the least water hungry crops good yields cannot be achieved. So desalination of sea water is a good way to obtain that water. Although technologies for water desalination are quite energy intensive, as water resources dwindle, they will start to become more economical. Spain already uses a fifth of its desalinated water for agriculture, although there are some problems with current technologies. Sodium and chlorine are removed, but desalinated water has too much boron (which hurts some crops) and its lack of magnesium, calcium and sulphate force farmers to use more fertilizers (Segal et al., 2006; Yermiyahu et al., 2007).

But although all these technologies help avoid problems caused by drought, sometimes even by using all modern technologies plants do not survive. So the best approach to fight the drought would include irrigation technologies, weather prediction and good planning, and breeding plants for drought tolerance.

1. 2. Breeding for drought tolerance

1.2.1. Breeding for water stress-response

A lot of advances have been done in the field of genetics during the last few decades. These advances can help in the breeding of plants to become more tolerant to drought and other abiotic stresses. New technologies also allow breeders to achieve their goals much faster, thus reducing the number of generations necessary to obtain an elite variety. Although traditional breeding has achieved many advances, nowadays the most used technologies for plant breeding are traditional breeding with marker assisted selection (MAS) and GMOs. However, GMOs' use in drought tolerance is limited, with only one variety in the market (Monsanto's DroughtGard, which expresses the CspB stress-response gene of bacteria). MAS has been successfully used in chickpeas to develop tolerant plants, and with sequencing technologies becoming cheaper, will be used more in the future (Eisenstein, 2013).

Some of the advances done by using MAS has even lead to succesful market release of seeds. In the CIMMYT, Mexico, a marker-assisted backcross (MABC) selection programme, meant to improve grain yield under limited water conditions, was carried out in tropical maize, and involved crossing a drought resistant line with a drought susceptible line. After the

backcrossing programme, five MABC-derived hybrids produced yields about 50% higher than those of control hybrids under severe drought conditions (Ribaut and Ragot, 2006).

Azucena, an upland japonica rice variety originally from the Philippines identified as drought tolerant (Courtois et al., 1996) was used as a donor parent to improve root morphological characteristics of Kalinga III, an Indian upland indica elite rice variety that escapes end-of-season drought through early maturity; yet this improved variety is still susceptible to early and mid-season drought (Steele et al., 2006). Again a marker-assisted back-crossing (MABC) breeding programme was used to pyramid four previously reported QTLs for improved root morphological characteristics (Price et al., 2000, 2002) from Azucena into Kalinga III. The resulting NILs were evaluated in field trials and four of them resulted superior in terms of tolerance (Steele et al., 2007). The result of this breeding programme was a highly drought tolerant rice variety, Birsa Vikas Dhan 111 (PY 84), released in the Indian state of Jharkhand (Steele, 2009).

Many of the drought tolerant plants found in the market right now were obtained by using a high-tech, MAS assisted method of traditional breeding. Syngenta's Artisian and Pioneer's AQUAmax corn strains were developed by using these techniques. Native corn genes were identified by using molecular data and selective crossbreeding helped to combine all those genes in a single plant. The plants were significantly tolerant to drought, and showed traits such as a reduction in the size of the stomata in the corn. However, most of the factors that made the plants tolerant are not known. This means a lot of fundamental research has to be done in order to find specific ways to improve drought tolerance (PIONEER, 2015; SYNGENTA, 2015).

Field trials necessary to test these hybrids are complicated and expensive; Pioneer, for example, tests the plants in more than 300 locations (PIONEER, 2015). And, although a lot of advances have been made in drought tolerance research (e. g., 2,594 on drought tolerance articles could be found in the Web of Knowledge database) that research often does not lead to a product in the market. Pioneer Hi-Bred International (DuPont), for example, tests thousands of hybrids every year, and only a few reach the market. This is because companies of this size have to market their product to fulfill the needs of farmers around the globe, so plants have to adapt to very different conditions and still give a good yield.

The expense of field trials means that plants that reach field trials should be as good as possible. So plants should be researched carefully to see how genes affects the phenotype and how the genes interact with each other and the environment. To start doing selection, the desired phenotype (plant characteristics) should be well known. The problem is that although the general objective may be known (e. g., improving yields in drought conditions) sometimes choosing the desirable phenotype characteristic is hard. For example, a variety of wheat developed in Australia that is more water efficient has a better yield than the control in drought conditions, but in presence of abundant water, its yield is substandard (Eisenstein, 2013). This is due to the high metabolic cost of these adaptations.

So developing traits for drought tolerance with a low metabolic cost is crucial, because, although drought tolerance gives the plants the ability to survive and produce offspring (seeds, fruit) even in unfavorable conditions the yield of the plant, even if it survives, may decrease significantly. For example, in root chicory, a plant that can withstand severe water deficiency in the last 3 months of its 6 month life, water scarcity still decreased root weight, leaf number,

total leaf area, and stomatal conductance, although water-use efficiency and leaf soluble sugar concentration increased (Vandoorne et al., 2012).

Another problem is the trade-off between the plants shutting down production during drought to survive and the death of the plant because it spent all its resources for fruit production. So choosing the right phenotype might be problematic, because a plant that gives a good yield under drought conditions may not give a good yield when water is abundant, or a mechanism for tolerance that is appropriate for certain heat and humidity conditions may not be appropriate for other conditions. But the breeder's job is to try to counterbalance all these opposing effects, making a plant that grows well in a certain environment. So, unlike resistances for biotic stresses, where one or a couple of genes will give resistance to that stress in any environment, although at different levels, tolerances to abiotic stresses depend on complex interactions between the genes and the environment.

Syngenta's Artesian corn hybrid, mentioned earlier, is a perfect example of what is desired. The hybrid matches or exceeds the yield of comparable hybrids in optimum growing conditions or under moderate stress conditions, and produces 13.7% more in severe drought conditions. In extreme conditions, the yield was 40% higher than the control (SYNGENTA, 2015).

The plants that are able to match the yields of other plants in normal conditions and produce more during water stress do not do it thanks to a single trait, but usually a combination of them, like in the case of the Artesian hybrid (SYNGENTA, 2015). There are a lot of traits that give tolerance to drought, and they are controlled by many genes, because they depend not on a single protein, but on the appropriate organization of organs, the production of osmolites, etc. Root structure, leaf area, stomatal conductance, and even the environment (soil structure, presence of subsoil waters, drainage...), all affect the resistance of a plant to drought. The response to drought affect the whole plant, and it has been shown that the main response to drought is the reduction of whole-plant transport capacity, coupled with a reduction in leaf area (reviewed by Maseda and Fernández, 2006).

The first response of a plant to a water shortage is to close the stomata, the organ though which a plant loses most of the water. But it has been shown in maize that plants that maintained stomata open and had active photosynthesis, are more tolerant to drought (Benesova et al., 2012). This is because an oversensitive response to drought does not let the plant to efficiently synthesize protective/detoxification proteins.

But, although all these traits are important, the organ that is responsible to reach the water and control its absorption is the root, so it is the most critical one for water acquisition.

Studies show that a stronger root system helps for drought tolerance (Shi et al., 2015) but a deeper root system is ineffective if there is no deep water. The water is provided by good irrigation, as mentioned earlier, and the improvement of plants makes it possible to reach the water. So, coupled with the other option mentioned earlier, improving plant roots for water stress response is critical in drought tolerance breeding.

1. 2. 2. Water stress response: Root adaptations

The root is the organ responsible for water and nutrient absorption, and without it the plant couldn't survive. There are a lot of factors that condition the amount of water absorbed by the root. Total root conductivity depends on the root length density in the soil (the number of contact points between water and soil) and the hydraulic conductivity of the root axe. But in order to absorb water, there must be water available for the roots, that is, there must be water at the root-to-soil interface. The interface appears when there is a flow of water towards the roots (well watered plants need only a small root mass to live), or the roots grow towards the water (Blum, 2011). The root-to-soil interface, however, tends to accumulate salts, making it harder for the plant roots to absorb nutrients (Stirzaker and Passioura, 2006).

So, in order to adapt well to environmental conditions, the plant roots need to grow with the appropriate root system architecture, towards the water. The root traits that give the root tolerance to abiotic stresses are called "traits for the second green revolution". The architectural traits that can be bred into the plants in this second revolution are traits that are under genetic control (have a good narrow-sense heritability) (Lynch, 2007).

As an example of traits under genetic control, genetic variation in root cortical aerenchyma (RCA) formation and secondary development in corn can reduce the metabolic costs of root growth and soil exploration. Thus, root respiration is reduced, as the cortical aerenchyma occupies the space of living tissue (cortical cells) with air, thus reducing the metabolic cost. This helped increase root length density in deep soil, increasing the drought tolerance of maize (Zhu et al., 2010).

Phenotypic traits can be defined in a broad (e.g., total root mass) or in a more specific way (distribution of the root mass depending on depth). More specific ways of measuring a trait can give a better understanding of the response, because two plants with the same root mass can have different root architecture, and can respond in completely different ways to the same stress. It has already been shown in rice that the critical part of the root for drought tolerance is the one at a 30 cm depth, where a good growth rate provides a good drought response index (Henry et al., 2011). This is also true in wheat, where partitioning the root mass to increase soil root density at depths of 60 to 120 cm instead of increasing root mass gives the plant greater tolerance to drought. A high root density at great soil depths is conditioned on having extensive root branching and long hair roots, which were shown to be determinant in drought tolerance by White and Kirkegaard (2010).

If the specific traits that are desired are known, selection via direct phenotypic evaluation or molecular markers is made easier, because conventional field screenings are costly and time-consuming (Lynch, 2007).

These traits can be inherited together or separately. For example, in a study done in melon (Fita et al., 2008) it was shown that root branching and root length can be inherited independently, making it possible to choose the desired characteristics. The independent inheritance of specific traits makes it possible to custom-design plants for specific environments.

In millet and sorghum, nodal and primary roots have a different response to water stress, depending on the species, and can also be selected independently. Because nodal roots are roots that are developed from the stem whenever the plant needs them, a rapid rate of development

and enhanced responsiveness may be traits that improve drought tolerance without affecting yield under optimum watering conditions (Rostamza et al., 2013). Traits that allow plants to react specifically to drought and improve yields during drought without affecting yields during well-watered times are really desirable. Traits that allow the plants to survive but have really small yields are not really desirable; the plant is grown for its yield, so having a good yield is the objective.

Traits associated to plant productivity under drought conditions are: small fine root diameters, long specific root length, and considerable root length density, especially at depths with available water. Not only root architecture is important in water uptake, though. Xylem pit anatomy helps to loose less water; RCA (root cortical aerenchyma) formation helps to waste less energy on cortical cells, allowing roots to grow deeper. A good deep growth rate and large xylem diameters in deep roots may also improve root absorption of water at depth. A xylem pit anatomy that would make it less prone to cavitation would improve yield under drought conditions without reducing yields when water is available. Rapid growth when water becomes available may improve tolerance to episodic droughts (Comas et al., 2013).

The rate of water uptake is also important, especially in climates were hot, sunny days make the plants use more water. It is regulated by the aquaporin activity and by suberin deposition (reviewed by Aroca et al., 2011).

Another interesting characteristic that helps plants withstand especially hot days when water is scarce is hydraulic lift. Hydraulic lift is the movement of water from roots to the dry top soil. Water absorbed by deep roots is released into the upper soil layers during the night, helping upper roots to survive and absorb water during the day, when plants need it more (Blum, 2011). In drought tolerant maize hybrids, this phenomenon helped to reach a peak transpiration rate 27–42% higher than the drought-susceptible hybrid on days where evaporative demand was high. The resistant hybrid had more primary roots is deep moist soil than the susceptible one (Wan et al., 2000).

But, even though root characteristics such as transport rate, metabolic waste, etc, are important, the most important one is the capability of the root to grow where the water is. If water is not available, it does not matter how good the plant is at keeping the water it has, or at transporting it; it would still die. That's why this works' primary objective is obtaining plants with a good root architecture.

1. 2. 3. Phenotyping the roots

Phenotyping roots is complicated by the fact that roots are hidden in the ground, and have to be taken out of the soil in order to measure them, which makes the continuous study of root growth complicated. However, a lot of techniques have been developed to deal with that problem, from using expensive MRI technologies (Schulz *et al.,* 2013) to creating transparent soil (Downie, *et al.*, 2012). A good and non-expensive way to phenotype roots may be the one used in a study done in melon, where roots were scanned after being grown in vertical agar plates (Fita et al. 2008). This system, though very artificial, allows observing the root growth and architecture in young plants, as agar simulates the hardness of the soil (Figure 1.2). In addition it has the advantage of being a very controlled system in which many plants can be

assayed at the same time. Inducing stress to plants grown in an agar plate is also made easy, because the medium can be easily controlled. For example, adding polyethylene glycol to the medium helps to stress plants (Penella et al., 2014). Polyethylene glycol is an osmolite that can reduce water availability, thus stressing roots.

Figure 1.2. Image of a one week old eggplant grown in an agar plate.

Hydroponics systems can also be used to grow the roots (Mathieu et al., 2015). However, hydroponics has a problem: because water moves the roots constantly, the secondary root branching (growth to the sides, no to depth) is not seen. In addition, the angle of the branching of each root is also lost.

1.3 The eggplant

The eggplant (*Solanum melongena* L., also called aubergine or brinjal), is a *Solanaceae* grown for its edible fruit. It is the third most produced *Solanaceae* in the world, after the potato and the tomato, with a world production of 49,418,212 tonnes (FAO, 2015). The main producers of eggplants are China and India, and the countries with the highest yields are the Netherlands and Belgium. Spain is the second producer in Europe, after Italy, and the 11th of the world (FAO, 2015). The fruit has to be cooked before eating, in order to get rid of its bitter taste. It is usually baked or fried, and sometimes marinated in vinegar, like the famous Almagro eggplants (a special protected variety of eggplant).

The eggplant is an herbaceous perennial plant with a deep root system, cultivated as an annual crop. As it originated from tropical climates, the eggplant needs warm temperatures to grow. The average temperatures it needs are around 20-30ºC during the day, and 15-20ºC at night, being able to withstand temperatures higher than 40ºC. The cold, however, is lethal for the eggplant, and it stops growing when temperatures are around 10-13ºC (Maroto, 1992). It also requires quite a lot of light and space, and in greenhouses, in crammed conditions can show symptoms of etiolation. The eggplant suffers from the same illnesses as other *Solanum*s, so it is not recommended to plant it in the field in less than four years after the other *Solanum* was planted (Maroto, 1992).

But, unlike other *Solanum*s, the eggplant does not come from America. The most recent theory about the origin of domestication of the eggplant says that the wild relative of the eggplant, *S. incanum*, came from Africa and the Middle East to Asia (Weese and Bohs, 2010), and the *S.*

melongena species was domesticated in the Indo-Birmanian region (Furini and Wunder, 2004; Hurtado et al., 2012; Knapp et al., 2013). It has been cultivated in the Asian southwest for a really long time, and it was brought to Europe in the Middle Ages, with the Arabs (Maroto, 1992).

Eggplants grow well in dry and hot climates. This quality makes them interesting for plant breeding for water stress tolerance. Some of the critical characteristics for drought tolerance, as shown in studies in *S. tuberosum*, are the strength of the root system, a high capacity for water absorption at later developmental stages and a higher leaf water content; which help to retain water (Shi et al.*,* 2015).

1. 3. 1. Taxonomy

The taxonomic description of eggplants began with Linnaeus, who described the main agronomic species of eggplant and its wild ancestor *Solanum melongena* and *Solanum insanum*, as well as the other two cultivated eggplant species, *S. aethiopicum* and *S. macrocarpon*, in his book *Species Plantarum*. The eggplants belong to the clade *Leptostemonum* (spiny *Solanum*), and there are three complexes of cultivated eggplants: the brinjal, or common eggplant complex (*S. melongena*-*S. incanum* complex) (Knapp *et al.*, 2013); the scarlet eggplant complex (*S. aethiopicum*-*S. anguivi*, and their intermediate forms), and the gboma eggplant complex (includes *S. macrocarpon* and *S. dasyphillum*) (Plazas et al., 2014).

Common eggplant complex

The common eggplant complex is characterized by its morphological plasticity, and there has been ample confusion on the classification of the melongena species. A lot of distinct plants were grouped into the species *S. incanum*, but later the species was found to be very diverse, including species *S. campylacanthum*, *S. lichsteinii*, *S. insanum*, *S. cumingii* and *S. melongena* (Samuels, 1996, Knapp *et al.*, 2013). Studies in chloroplast genomes have further confirmed the distinction of *S. lichsteinii* (Sakata and Lester, 1994).

Solanum melongena L. (Figure 1.3, J, K and L), is cultivated worldwide, with a great diversity of cultivars in Asia, and secondary diversity centers in the Middle East and the Mediterranean. *Solanum melongena* crosses well with its ancestor *S. insanum,* and gene flow between these species still occur (Knapp *et al.*, 2013). It has been suggested by Meyer *et al.* (2012) that it was domesticated twice. The nomenclature of *S. melongena* is complicated, because eggplants with different fruits have been named differently. But, although the fruit size, shape and taste vary a lot, it is still the same species, including only cultivated plants (Knapp *et al.*, 2013).

S. incanum, since it was defined by Linnaeus*,* has been confused with *S. insanum, S. coagulans,* and *S. campylacanthum*. It is a species that grows in dry areas, from northern Kenya to Pakistan, and grows in drier areas than other eggplants, making it a good source of genes for dorught tolerance, because it crosses well with *S. melongena*. It is morphologically similar to *S. lichsteinii*, and clusters with it in phenetic analyses, but the geography and its less corrugated stem make them different (Knapp *et al.,* 2013). It is thought that *S. incanum* is the

ancestral type, form which *S. melongena*, *S. campylacanthum* and *S. lichsteinii* originated (Lester and Hasan, 1990).

S. insanum (Figure 1.3 D, E, and F) grows at low elevations, from India to SE Asia, and can also be found in Madagascar and the Mauritius. The circumscription of this species includes wild progenitors and feral "reversions" from the cultivated form (Daunay et al*.,* 2001). Some consider it as the wild ancestor of the cultivated eggplant (Daunay et al. 2001), in Asia. *S. insanum* and *S. melongena* are highly interfertile, so introgression between these species sometimes makes it difficult to assign plants to a species.

Solanum lichtensteinii (Figure 1.3 G, H and I) grows from South Africa to Tanzania, at 500-2000 m. It is morphologically similar to *S. incanum*. Plants in upland dry areas of South Africa can be very small, with reduced leaves, while there are not known dwarf forms for *S. incanum* (Knapp *et al.*, 2013).

Solanum linnaeanum grows in the Mediterranean region, although it is thought it originated in South Africa, at elevation of 0-1200 m. It is also known as *S. sodomaeum* L. or *Solanum hermannii* Dunal, the second name being rejected by the rules of nomeclature. It is morphologically distinct from other eggplants, because of its deeply incised, almost glabrous leaves. *S. linnaeanum* is an important source of resistance to various diseases (Knapp *et al.*, 2013).

Scarlet and gboma eggplant complex

The gboma eggplant complex and the scarlet eggplant complex are common to Sub-Saharan Africa. The scarlet eggplant is one of the most important vegetables in West and central Africa (Maundu et al., 2009). The gboma eggplant, on the other hand, is less important, although it is one of the major crops in Benin, rain forest areas of Coastal Africa, and in West Africa (Lester et al*.,* 1990). Both complexes are a major source of diversity for the cultivated eggplant.

S. dasyphyllum is the wild ancestor of the domesticated *S. macrocarpon*. They are completely interfertile, and weedy varieties are formed by inter-species hybrids (Bukenya and Carasco, 1994).

S. anguivi (Figure 1.3 A, B and C) is the wild ancestor of *S. aethiopicum* (Lester and Niakan, 1986), and inter-species hybrids are completely fertile (Lester and Niakan, 1986; Lester and Thitai, 1989). *S. anguivi*, as well as a cultivar of *S. aethiopicum* used only for its leaves, has small fruit size (Plazas et al., 2014).

Related species of cultivated plants usually are good reservoirs of genes of interest for breeding, as shown in studies where eggplant was grafted in wild species rootstock (Gisbert et al., 2011). The wild eggplants that come from Africa could be a really interesting source of diversity in order to obtain traits that make plants tolerant to drought, because of the arid climate of Central Africa. However, the different species of eggplant have certain degree of incompatibility problems, as shown in a study done by Bletsots et al. (1998), where 3 accessions of *S. melongena* were crossed with *S. torvum* and *S. sisymbriifolium* (wild species of eggplant), and only *S. melongena x S. torvum* hybrids were obtained. Furthermore, the F1 plants only gave fruit when crossed with *S. melongena*. .Therefore sometimes it is difficult to obtain hybrid seed. In addition, these seeds also have problems to germinate. The group of

eggplant breeding of the COMAV-UPV have performed a series of interspecific crosses in order to introgress genes from wild relatives to cultivated eggplant, and some of these hybrids are going to be used in this work (see materials and methods).
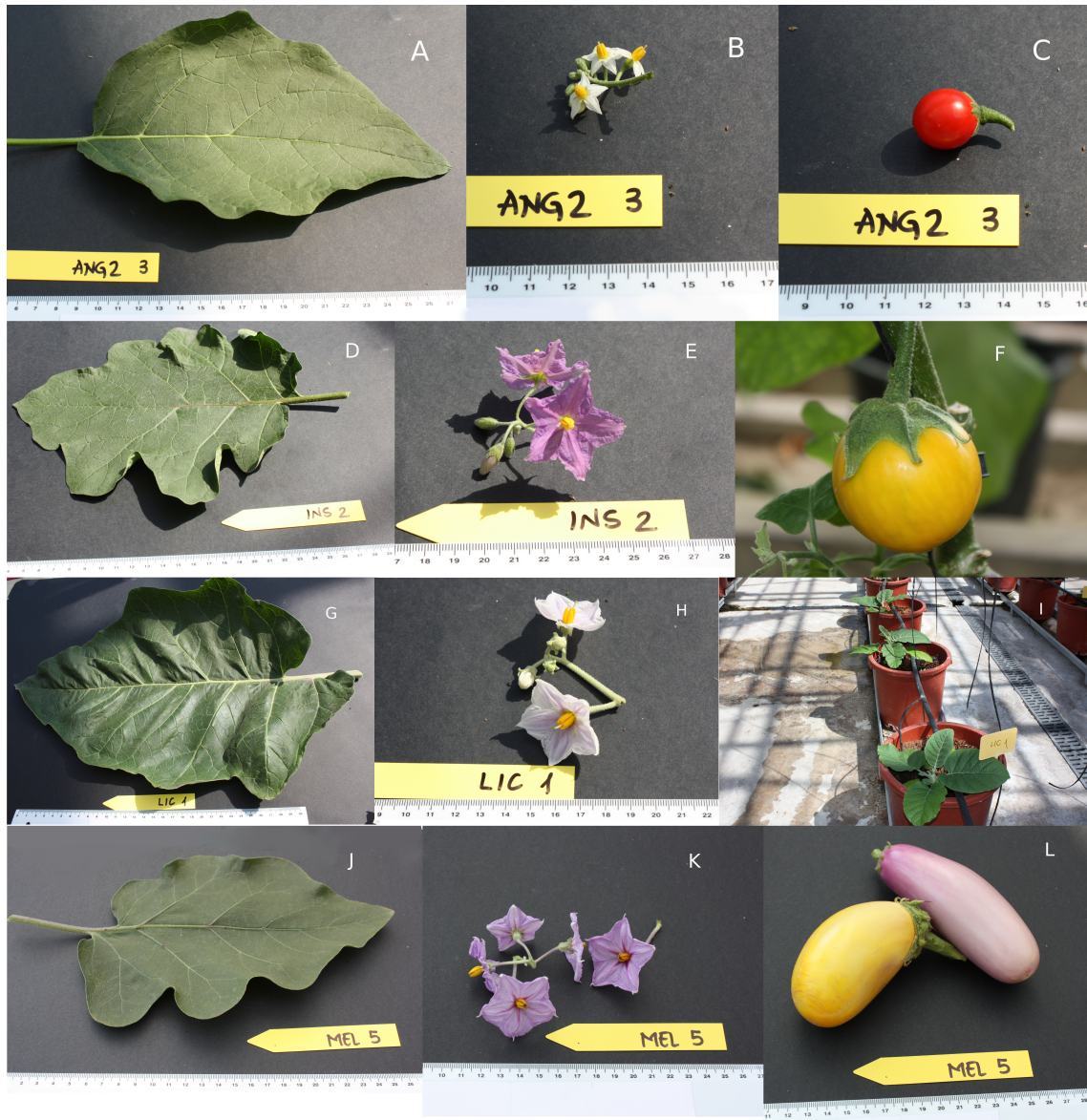

Figure 1.3 A, B, C, photos of *S. anguivi* leaves, fruit and flower, respectively, D, E, F, images of *S. insanum* leaves, flowers and fruit, G, H, and I are images of *S. melongena*, and J, K, and L are images of a *S.melogena x S. insanum* hybrid.

1. 3. 2. Eggplant response to water deficit

Eggplant is more tolerant to drought than other vegetables, and has a better stomatal control on transpiration Behboudian, 1977). Unfortunately there are not many studies on the genetics of such tolerance or the value of wild relatives in the improvement of such character. Karan et al (2011) reported that an eggplant with good irrigation could have a yield of 33.7 t/ha, while plants watered at 80, 60 or 40% of their total field capacity have yields that were 12, 39 and 60% less. On the other hand, fruit dry matter content and water productivity, the fruit dry weight obtained per unit of water used increased. Interestingly, deficit irrigation 2 weeks before

flowering saved as much water as the 80% treatment, with the least yield reduction, making that kind of watering in drip-irrigation systems optimal (Karam et al., 2011).

Eggplants have been improved for drought tolerance by using grafting (Zhou et al., 2010). Genes and landraces useful for breeding have been identified (Sudarmonowati et al., 2012), and a transgenic approach has also been used. A transgenic eggplant variety carrying the bacterial mannitol-1-phosphodehydrogenase (mtlD) gene germinated better and responded better to salinity than the untransformed variety. However, the improvement of eggplants against drought is still on its early stages, and much work is still to be done.

In previous works by the group the tolerance to drought was recorded in 3 accessions of Solanum melongena and in some wild relatives (Fiorucci, 2014). However hybrid and wild species seeds showed quite a lot of problems to germinate, so a study seeking to improve the germination of eggplants was done.

1. 4. Seed germination

S. melongena and related species have seed dormancy and sometimes have difficulties to germinate. Quick and uniform germination of the seeds is beneficial for breeding (allowing to use the seeds just recovered by the fruit) and phenotyping in juvenile stages (when differences in germination may affect the results). A lot of plants, especially non-cultivated species, have a mechanism called seed dormancy that doesn't allow the seed to germinate until certain conditions have been met. The dormancy can be either physical (e.g., a water-impermeable seed coat) or physiological (e.g., low growth potential of embryo). This mechanism exists so that plants don't start to grow in conditions that would harm them, like winter cold temperatures. For example, in *S. rostratum*, a weed, dormancy is broken by cold stratification during winter and early spring, so it germinates in late spring (Shalimu et al., 2012). In *S. nigrum* and *S. physalifolium*, also weeds, low winter temperatures weakened dormancy and high temperatures strengthened it, because seeds need a quantity of cold days to germinate (Taab and Anderson, 2009). In *S. viarum*, cold stratification during 14 days improved the percentage of germination compared to the control (Kandari et al., 2011)

In the case of S. melongena just the passage of time improves germination greatly. This could be due to the reduction of abscicis acid (ABA) levels on the seed. Abscisic acid plays a big role in seed dormancy, as well as dormancy of terminal buds in preparation for winter, among other things. In *S. melongena*, non-dormant cultivar 2-month old seeds had a concentration twice as low as 2-month old seeds of dormant cultivars. ABA concentration was considerably reduced in 12 months in dormant seeds (Yogeesha et al., 2005).

There are treatments that can reduce germination time, allowing to grow a plant immediately after obtaining a seed. This is of a great interest in breeding programmes, where being allowed to grow multiple generations within a year reduces the cost of the program.

Soaking young seeds in gibberelic acid (GA_3) removes seed dormancy, probably reducing ABA concentration (ABA and GA have antagonitstic effects in seed dormancy) (Yogeesha et.al, 2006). GA_3 has an interesting effects in seed germination, due to its antagonist function with ABA. *S. saccharroides* seeds only germinate when a certain thermal time has been given

(seeds have received a quantity of heat during a number of days), but a high GA_3 concentration reduced thermal time (Monte *et al.*, 2010). In another study done in *S. rostratum*, gibberelic acid also broke physiological dormancy (Wei *et al.*, 2010), thus reducing germination time.

KNO_3 also has an effect in breaking seed physiological dormancy in *S. rostratum* (Wei et al., 2010), and *Arabidopsis thaliana* (Batak et al., 2007). KNO_3 acts on seed dormancy by being a nitrous oxide donor, a molecule involved in seed germination (Batak et al., 2007).

Soaking seeds in water is a highly efficient way of breaking physical or physiological dormancy. This has been proven in studies done in *S. aculeastrum* (Adebola and Afolayan, 2006); *Momordica charantia* (Saleem et al., 2014); *Asparagus acutifolius* (Conversa and Elia, 2009). This makes sense, because imbibition of seeds by water is an indispensable thing for seed germination. However, not only water is necessary; sometimes, the absence of some key nutrients in the solution may have an effect on seed germination, such as the absence of nitrogen or phosphorus (Kandari et al., 2011).

Physical dormancy can be broken using other physical methods, such as sandpaper scarification, H_2SO_4 scarification, and bleach scarification. H_2SO_4 and sandpaper scarification were found to increase germination rates in *Solanum viarum*, a tropical wild shrub (Kandari et al., 2011). H_2SO_4 is not only effective in breaking dormancy; it also is a powerful disinfectant (Pandrangi et al., 2006), which may make it useful for *in vitro* culture of seeds. Bleach is also used for disinfecting seeds for *in vitro* culture.

Solanum melongena is the eggplant that is cultivated the most in the world. So it is the species that is best germinates in human made conditions. During our studies, our group has observed that wild eggplants, such as *S. dasyphyllum* and *S. anguivi* do not germinate very well *in vitro*. *S. insanum* germinated better, but still had problems. Even *S. melongena* does not germinate really well *in vitro*, or on Petri dishes. So the study on the improvement of germination of *S. melongena,* using factors that could be used *in vitro*, such as soaking, GA_3, etc., will help to germinate seeds in vitro, and even though species such as *S. anguivi* are clearly distinct from *S. melongena*.

2. OBJECTIVES

This work is part of a higher project aimed at introgress traits of interest such as a tolerance to drought from wild relatives to eggplant. Taking into account the problems observed by the group in the germination of the seeds and the importance of the root system in the drought tolerance response.

The main objectives of this project were:

1. Obtain a suitable protocol for rapid eggplant and wild-relatives seed germination.

2. Develop a protocol to evaluate water stress in young plants of *S. melongena* and study the root architecture of *Solanum melongena*, *S. anguivi*, *S lichsteinii* and their hybrids under normal and drought conditions.

3. MATERIALS AND METHODS

3.1 Obtaining a suitable protocol for rapid eggplant and wild-relatives seed germination

3.1.1 Seed materials and germination conditions

To obtain a suitable protocol for rapid eggplant and wild-relatives seed germination an experiment testing different treatment combinations on fresh and old seeds of *S. melongena* was performed. The accession Mel 5.3 (corresponding to Almagro Eggplant) of which there was a lot of seeds available was chosen for the experiment. Fresh seeds of Mel 5.3 () were extracted from physiologically ripe eggplants grown in the open field at the Universitat Politècnica de València (Valencia, Spain). Five years old seeds of the same eggplant accession, were also used as a control to see the effect of the treatments on seed dormancy. Although the root phenotyping experiment (see section 3.2) was aimed to be done with several wild relatives of *S. melongena*, for the seed germination experiment we chose *S. melongena* seeds because a high quantity of seeds was required (1200 seeds) for the experiment, and such an amount was not available for these species, such as *S. anguivi.*

Seeds were germinated in Petri dishes (8.5 × 2.5 cm) on a layer of 0.5 cm of hydrophilic cotton covered by filter paper (Figure 3.1). 25 seeds were seeded in each Petri dish (Figure 3.2). In the beginning of our experiment (day 0) the Petri dishes were placed in a climatic chamber with a 16 h light / 8 h darkness photo period and a 25 ºC temperature. GRO-LUX F36W/GRO (Sylvania, Danvers, MA, USA) fluorescent tubes gave the light. The humidity in the dishes was kept constant by covering them with a lid and watering if necessary.

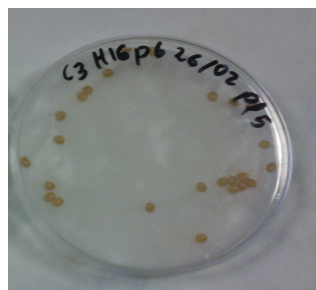Figure 3.1. Image of the plates used with cotton and the filter paper.

Figure 3.2 Image of plate with non germinated seeds.

3.1.2 Factors evaluated and experimental design

In order to test the effect of each factor on germination an orthogonal array design was used. This kind of design reduces greatly the number of combinations that have to be tested in order to find simple effects.

Seven factors, named Soaking, NaClO, GA_3, KNO_3, Cold, Heat, and Light, with two possible levels (level 0, L_0; level 1, L_1) for each factor, were used to evaluate their effect on seed dormancy in *S. melongena*. The levels for each treatment are defined as following:

a) Soaking:

L_0= no soaking;

L_1=soaking seeds in water for 1 d.

b) NaClO:

L_0= no NaClO scarification;

L_1=NaClO scarification by immersion of seeds for 10 min in a 30% NaClO solution followed by rinsing seeds with running water for 3 min.

c) GA_3:

L_0 = no GA_3 application (Gibberelic acid);

L_1=soaking seeds in a 500 ppm solution of GA_3 for 1 d.

d) KNO_3:

L_0= use of deionized water as a moistening agent in the Petri dishes;

L_1=use of a 1000 ppm KNO_3 solution for watering the Petri dishes the first time (moist was kept by watering with water).

e) Cold;

L_0= no cold stratification;

L_1= cold stratification applied by placing seeds on Petri dishes with a moistening agent at 4ºC for 7 d.

f) Heat;

L_0= no heat shock;

L_1 =placing seeds on Petri dishes with moistening agent at 37ºC for 1 d.

g) Light:

L_0=seeds placed in darkness (Petri dishes covered with aluminum foil) at day 0; L_1=seeds subjected to light irradiation (16 h light / 8 h dark) at day 0.

Factors Soaking, NaClO and GA_3 were applied before placing seeds on Petri dishes or sowing them in nursery growing substrate. Factors KNO_3, Cold and Heat were applied after placing

seeds on moistened Petri dishes sowing, but before initiation of the evaluation of germination or emergence (day 0). The Light factor was applied at the initiation of the experiment (day 0).The factors were given in the following order: 1) Soaking, 2) NaClO, 3) GA_3, 4) KNO_3, 5) Cold, 6) Heat, and 7) Light. As factors in the pre-germination procedures may take up to 7 days, and the longest combination of L_1 factors was 9 days, some treatments started up to nine days before the initiation of the evaluation of germination emergence (day 0).

The main effects of the seven factors studied at two levels were evaluated using an L8 (27) orthogonal array design (Roy, 2010) consisting of eight treatments (Table 3.1). These eight treatments are orthogonal and each of the two levels (L_0 and L_1) for each factor is represented in the different treatments the same number of times (four), of which for any other factor one half (two) are evaluated at level L_0 and the other half (two) are evaluated at level L_1 for any other factor. For each treatment, six replicates (six Petri dishes) were used. Significance of differences among treatment means was evaluated by doing an ANOVA with a level of significance F=0.05

The degrees of freedom and sums of squares of the ANOVA for the eight treatments were partitioned in seven orthogonal contrasts for testing the significance of the main effects (i.e., the difference in the average between levels L_0 and L_1) for each factor (Little and Hills, 1978).

Table 3.1. L8 orthogonal array matrix (2^7) for the seven factors evaluated (Soaking, NaClO, GA_3, KNO_3, Cold, Heat, and Light) at two levels (L_0 and L_1), indicating the levels applied to each of the eight treatments tested. The day of beginning of initiation of application of the different levels in order to have a synchronization of initiation (day 0) of the germination experiment is indicated.

	Factors							
Treatments	Soaking	NaClO	GA_3	KNO_3	Cold	Heat	Light	Day of initiation
1	L_0	L_0	L_0	L_0	L_0	L_0	L_0	0
2	L_0	L_0	L_0	L_1	L_1	L_1	L_1	-8
3	L_0	L_1	L_1	L_0	L_0	L_1	L_1	-2
4	L_0	L_1	L_1	L_1	L_1	L_0	L_0	-8
5	L_1	L_0	L_1	L_0	L_1	L_0	L_1	-9
6	L_1	L_0	L_1	L_1	L_0	L_1	L_0	-3
7	L_1	L_1	L_0	L_0	L_1	L_1	L_0	-9
8	L_1	L_1	L_0	L_1	L_0	L_0	L_1	-1

3.1.3. Traits evaluated

Seed germination was evaluated at 7 and 15 d after initiation of the germination experiment (day 0). For a seed to be considered germinated the radicle had to be 1 mm or longer. The total amount of germinated seeds was counted.

3.2. Eggplant root phenotyping

3.2.1 Experimental design and plant material

To evaluate the root architecture seedlings were grown in agar plates, using the method described previously by Fita et al. (2008) to phenotype the roots. Two different treatments

were given, one to simulate drought conditions (water stress) by using PEG (polyethylene glycol) and control conditions with normal in vitro media. Before starting the assay, several tests to optimize the PEG level and find the most transparent kind of gel were done (explained in sections 3.4.1 and 3.4.2). As plant material 9 genotypes were tested (*S. melongena, S. anguivi, S. lichsteinii, S. dasyphyllum, S. insanum, S. melongena* x*S. anguivi, S. melongena* x *S. lichsteinii, S. melongena* x *S. dasyphyllum S. melongena* x *S. insanum*).

3.2.2 Determining the optimum polyethylene glycol concentration

In order to evaluate the optimum level of stress for the eggplant, plantlets with 1 cm radicles were placed in methacrylate plates with 19 cm × 1 cm × 23 cm measurements, using 0, 3% and 7% of Polyethylene glycol concentrations to determine the appropriate one.

In order to germinate, seeds were placed in sterile agar plates with the following concentrations of nutrients: 15 g/L of D (+)- Sucrose (Panreac, Applichem), 4.3 g/L of Murashige and Skoog salts with vitamins (Duschefa Biochemie, Netherlands) and 8 g/L of industrial agar.

Plantlets that grew 1 cm were placed in the 19 cm × 1 cm × 23 cm sterile plates, which had a gel with the following concentration: 30 g/L of D (+)- Sucrose (Panreac, Applichem), 4.3 g/L of Murashige and Skoog salts with vitamins (Duschefa Biochemie, Netherlands) and 6 g/L of Phytagel (Sigma-Aldrich). Plates also had polyethylene glycol (mol wt.8, Sigma-Aldrich) at the following concentrations: 0, 3% and 7%.

All work was done *in vitro* (the plates, all instruments and the gels were sterilized in the autoclave, while the seeds where disinfected with bleach). The plates were covered with hydrophobic cotton and aluminum foil, and all work was done in a flow cabin. In front of a burning light (Figure 3.3).

3.2.3. Agar transparency evaluation

In order to scan the roots properly, the background has to be different enough from the roots.

Three 19 cm × 1 cm × 23 cm plates were done, which had the same concentration as above, but instead of Phytagel, the gelifying substances tested were: Industrial agar (Pronadisa, Conda), Gelrite (Duschefa Biochemie, Netherlands) and Phytagel (Sigma- Aldrich), at 6 g/L concentrations.

3.2.4. Eggplant root growth

19 cm × 1 cm × 23 cm methacrylate plates were used to plant the seeds (Figure 6). The sterile plates were filled to half the height, with a gel that had the same concentrations as in point 2.4.1, but one substance was added to improve germination *in vitro*: KNO_3 (without anticaking agent, Panreac Quimica, Spain) at a 1000 ppm concentration. Polyethylene glycol (mol wt.8, Sigma-Aldrich) was added to the plates that were going to have water stress treatment, at a 3% concentration, and the control had no polyethylene glycol.

Four seeds were placed in each plate, two treatments were done per genotype, and 4 plates/genotype (16 seeds, 2 treatments). Seeds were placed in sterile conditions, after keeping them for 10 min at a 30% bleach solution, and washed by keeping them for 10 min in sterile water 3 times. The plates were closed by hydrophobic cotton and covered with aluminum foil.
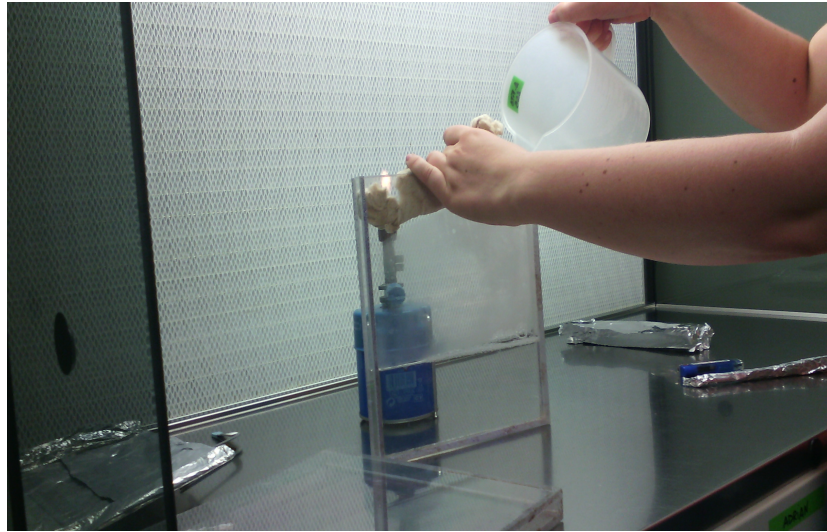

Figure 3.3 Figure of how the gel in the plates is poured, in a flow cabin.

The genotypes tested were: *S. melongena, S. lichsteinii, S. melongena*S. Lichsteinii, S. anguivi, S. melongena*S. Anguivi, S. Insanum, S. melongena*S.insanum. S. dasyphyllum* and *S. macrocarpon* and their hybrids were also used, but did not germinate.

3.3. Root growth analysis

The plants in the plates were scanned using an Expression® 1640XL scanner (Seiko Epson Corporation; Nagano, Japan) (Figure 3.4), at a medium (400 dpi) resolution. This images were evaluated using WinRHIZO Pro 2.3. (Regent Instruments Inc., Quebec, Canada) (Figure 3.5 and 3.6). This program measured the average diameter, the total length of the roots, the length of roots of a certain diameter, and the number of tips. Root architecture was also evaluated by hand by using Gimp 2.8 whether it was straight and deep, medium width and deep, wide and deep, or wide and superficial.

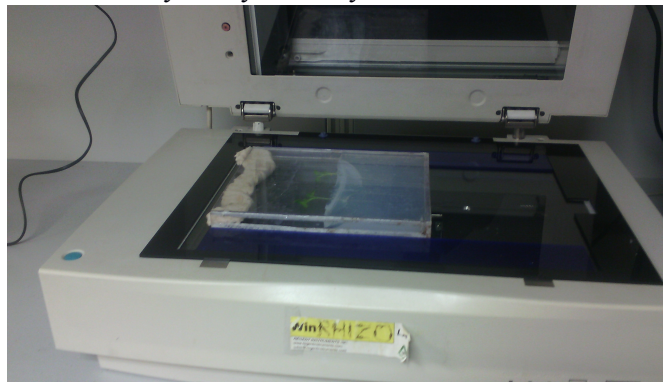The data obtained was analyzed by one-way ANOVA tests.

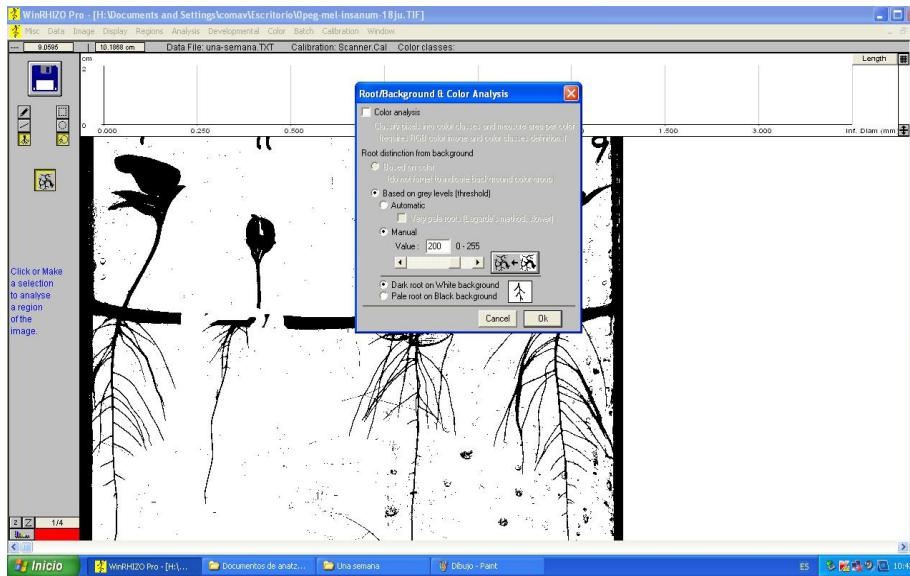Figure 3.4 The Epson Expression scanner.

Figure 3.5 Images of roots were manually adjusted so the contrast between the background and the roots was the right one.
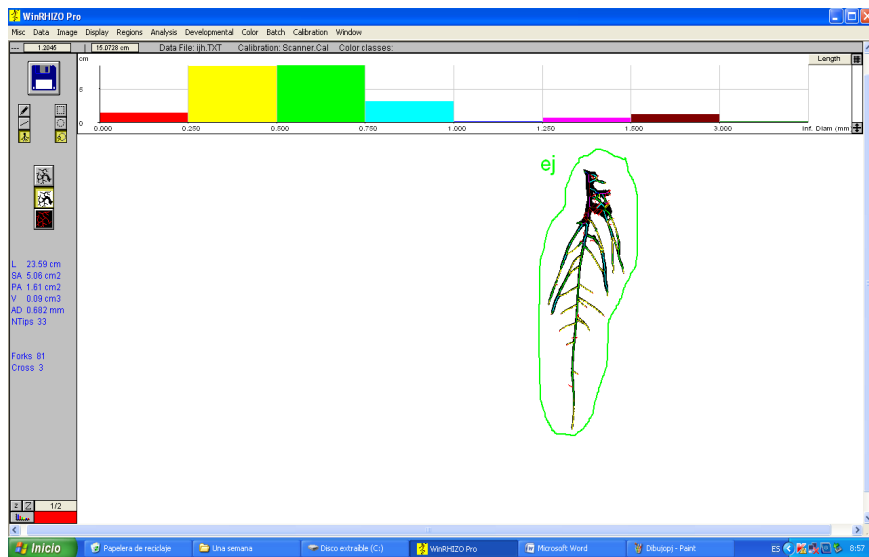

Figure 3.6 Image of how the program sees the roots.

20

4. RESULTS AND DISCUSSION

4. 1. Obtaining a suitable protocol for rapid eggplant and wild-relatives seed germination

The amount of germinated seeds from old and fresh seeds of each plate and treatment was recorded every day. The ANOVA analysis of the results (Table 4.1) showed that there were significant differences among old and fresh seeds in terms of days to first seed germination, and number of germinated seeds in a certain day (i. e. 7 d and 14 d). The different combinations also affected germination significantly, and there was a significant interaction between the combination and age of the seed (Table 4.1).

Table 4.1 Multifactorial ANOVA analyzing the effects of the treatment combination tested and the seed age.

	d.f.	Mean Square Seeds germinated 7d	Seeds germinated 14d	Days to first germination
MAIN EFFECTS				
Combination	7	1055.85***	1525.15***	3*
Seed age	1	522.67***	518.01***	19.67***
INTERACTIONS				
Combination x Seed age	7	141.91***	209.42***	1.89***
RESIDUAL	80	10.01	1.14	0.45

d.f: Degrees of freedom; ^{Ns}, *, **, ***, mean non-significant, P-value < 0.05, 0.01, and 0.001 respectively [1]: because seeds did not germinate at all in some treatments, and null data cannot be used for the ANOVA analysis, only C2, C5, C6 and C8 were used.

Some combinations were able to germinate seeds really soon for example, seeds subjected to combination 6 germinated at day 3, whereas other combinations, such as C3 or C4 failed to germinate almost any seed, (C4 germinated just one seed at day 14; Figure 4.1). Interestingly, combinations C1 (C1 is the control one, and did not have any of the factors studied) and C7 (includes soaking, bleaching, cold, heat and darkness) helped to germinate old seeds but failed to germinate fresh seeds (Figure 4.1 and 4.2), and combination 2 (with KNO_3, cold, heat and light) resulted in faster germination in fresh seeds rather than old ones. The differences in terms of response to the combinations among fresh and old seeds could be explained by the fact that fresh seeds still have physiological dormancy. Generally, old seeds germinated better, with an average germination rate for all treatments of 13.85 seeds per plate while it is 9.77 seeds per plate in fresh ones (Figure 4.3).

The number of seeds germinated did not change too much from 7 d to 14 d (Figure 4.3); combinations that worked well reached rapidly a hundred percent germination rate and combinations that did not work at 7 d had no significant change at 14 d. The average germination rate did not change much either. Furthermore, the objective of this experiment was to accelerate germination, so combinations that helped to germinate a great proportion of seeds within a week are more desirable. For this reason, the simple effects of each treatments and combination are analyzed just for 7d in the next section.
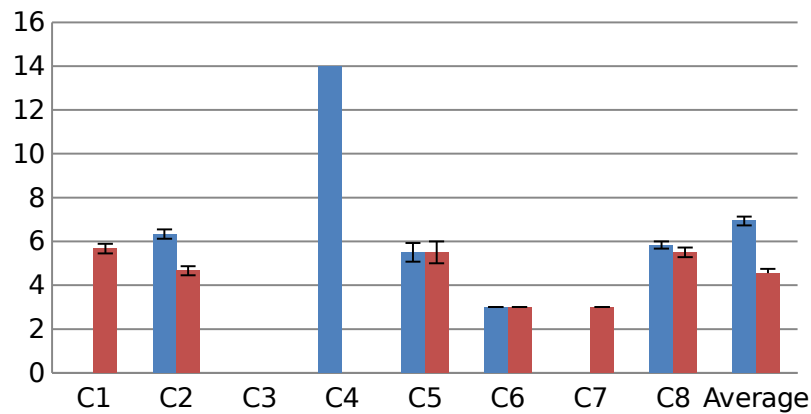
Days to first germination

Figure 4.1. Bar chart showing the number of days it took in each combination to germinate at least one seed. Blue bars correspond to fresh seeds and red bars to old seeds. The lower the bar, the better (less days to germinate). Error bars correspond to the standard error.

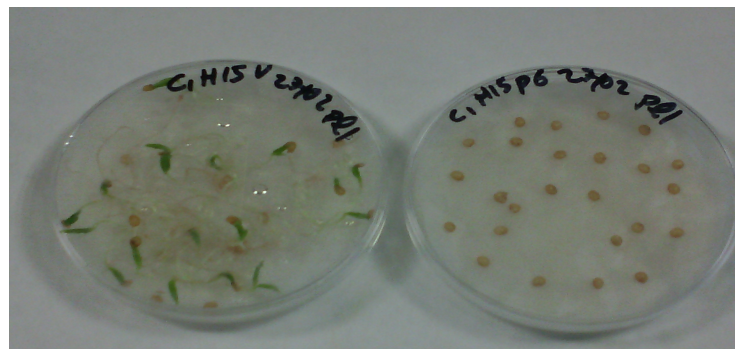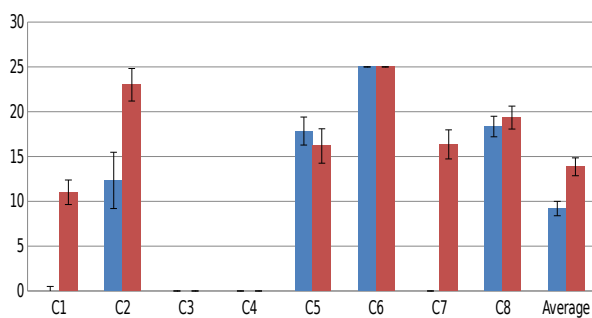Figure 4.2. Plates with combination 1, after a week, in old seeds (left) and fresh ones (right).

Number seeds germinated day 7
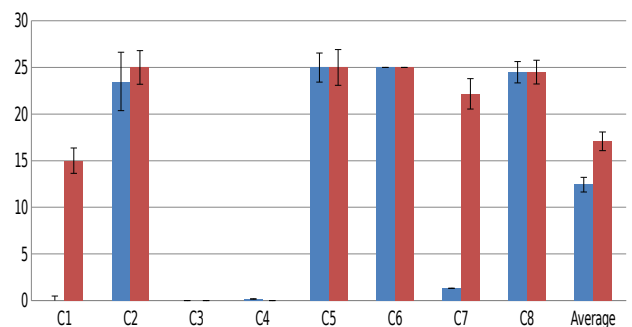

Number seeds germinated day 14

Figure 4.3. The number of seeds (out of 25) that germinated, on average, for each combination at day 7 (left) and day 14 (right). Blue bars correspond to fresh seeds and red bars to old seeds. Error bars indicate the standard error.

4.1.1 Simple treatment effects on fresh seeds

In table 4.2 the combination effect on the number of fresh germinated seeds at day 7 can be observed. For fresh seeds, combination 6 (soaking, GA_3, KNO_3, heat and darkness) clearly stands out with a 100% germination rate (Table 4.2) and a very quick germination as we explained before. The combination effect, calculated as the average germination rate of the combination minus the overall average was the highest for C6. The other combinations which had an positive effect were in decreasing order C8 (soaking, bleaching, KNO_3, and light), C5 (soaking, GA_3, cold, light) and C2 (KNO_3, cold, heat and light). . Combination 1 (no treatment), 3 (bleaching, GA_3, heat and light), 4 (bleaching, GA_3, KNO_3, cold) and 7 (soaking, bleaching, cold, heat and darkness) had negative effects on germination. .

Table 4.2. Number of seeds germinated out of 25 in each plate in fresh *S. melongena* seeds, the average amount and the combination effect.

Combinations	Total number of seeds germinated	Average per plate	Combination effect
1	0	0	-9.77
2	102	17	7.23
3	0	0	-9.77
4	0	0	-9.77
5	107	17.83	8.06
6	150	25	15.23
7	0	0	-9.77
8	110	18.33	8.56

The ANOVA analysis of the simple effects of the treatments in the different combinations, showed that soaking, KNO_3, and light had significant positive effects on germination, whereas bleach and cold had significant negative effects. Surprisingly, GA_3 did not show a significant effect along with the heat treatment (Table 4.3).

Table 4.3. Effect of each treatment, its degrees of freedom and its F values for fresh *S. melongena* seeds.

Treatment	Simple effect	d.f.	F value
SOAK	11.04	1	3.25×10^{-13}
BLEACH	-10.38	1	2.01×10^{-12}
GA3	1.88	1	0.0787
KNO3	10.63	1	1.01×10^{-12}
COLD	-2.13	1	0.047
HEAT	1.46	1	0.17
LIGHT	7.04	1	3.83×10^{-8}

Favorable combinations (6, 8 and 5) have in common the most critical and significant (Table 4.3) factor: soaking. This makes a lot of sense, because, as it is explained before, soaking helps germination in a lot of species. Imbibition of seeds by water is a required factor for germination, although there can be more factors (Koo et al., 2015). It is quite clear that although soaking is not the only significant factor with a positive effect, it is the one with the highest effect (it has the highest simple effect value). The two best combinations also share

another factor with a significant positive effect: KNO_3, which as was explained before, acts as a nitrous oxide donor, a molecule that has a role in signaling during germination (Batak et al., 2007).

The huge negative effect which bleach has on germination rate can be noticed in the difference between the combination effect of C6 and C8. These two treatments differ just on bleach and light. Light seems to have a positive significant effect, although a small one, but the effect of bleach counteracts it, making C8 worse than C6, although C8 has light and C6 does not. Light is also a regulator in germination, whose absence can be compensated by heat shock, as shown in studies in tobacco (Koo et al., 2015). Bleach, though, acts as a scarification agent. The germination could possibly improve if sulphuric acid was used instead of bleach for disinfection (necessary for *in vitro* culture), as shown in studies in *Solanum viarum* (Kandari *et al.,* 2011). The effect of bleach can also be seen in combinations 3 and 4, where seeds did not germinate at all (this may be because the seed were dry, absorbed very concentrated bleach, and the seed embryo died).

So, as the analysis shows, the best combination would be C6 with a light treatment. The heat, cold and GA_3 treatments could be omitted, because they seem to have no significant effect. The treatments used would be: soaking, KNO_3 and light. But as our work has to be done *in vitro*, in sterile conditions, we would still need to use bleach (like in C8).

4.1.2 Simple treatment effects on old seeds

In old *S. melongena* seeds, combination 6 was also the best, followed by combination 2 and combination 8 (Table 4.4). Here, in contrast with the fresh seeds, combination 5 did not have a very good effect. And combination 7 resulted positive to germination. This happened because in dry *S. melongena* seeds the factors that were significant were different: GA_3 and heat were significant, while light was not (Table 4.5). Bleach and soaking are still significant in old seeds. C6 is so good because it combines all the treatments with a positive significant effect (and has GA_3, which has a negative effect in this case), while C2 only had the KNO_3 and the heat treatment out of the significant ones. C8, although it suffers from the huge negative effect of bleach, still does fairly well, due to the positive effect of soaking and KNO_3. So, the significant and positive effect factors are soaking, KNO_3 and heat, while GA_3 and bleach have a significant negative effect. Cold and light are not significant.

Table 4.4. Number of seeds germinated out of 25 in each plate in old *S. melongena* seeds, the average amount and the combination effect.

Combinations	Totals	Averages	Combination effects
1	66	11	-2.85
2	138	23	9.15
3	0	0	-13.85
4	0	0	-13.85
5	97	16.17	2.31
6	150	25	11.15
7	98	16.33	2.48
8	116	19.33	5.48

GA$_3$, according to the literature, has a positive effect on germination (Monte et al., 2010; Wei et al., 2010). However, the analysis of the data shows that the effect in this case is negative. The reason why is unknown. It could be because, as the seeds used are old ones, where physiological dormancy has already been broken by the passage of time, excess GA$_3$ affects germination negatively. It could also be because of the Soaking x Bleach interaction mask the effect of GA$_3$ (Table 4.6). There are also other interactions masked by simple effects: cold masks the Soaking x KNO$_3$ interaction, light masks the Soaking x Heat interaction, heat masks the Bleach x KNO$_3$ interaction.

Another problem is the huge effect of bleach. In combinations 3 and 4, the seeds did not germinate at all, because the seeds were introduced in bleach, and only after that did the seeds receive the other treatments. So the seed embryo may have been killed by the bleach, thus canceling the effect of the other treatments. Changing the order and putting the GA$_3$ treatment before the bleach treatment would let us know whether the effect of GA$_3$ is really negative on old seeds.

Table 4.5. Effect of each treatment, its degrees of freedom and its F value for old *S. melongena* seeds.

Treatment	Simple effect	d.f.	F value
SOAK	10.71	1	1.23×10^{-14}
BLEACH	-9.88	1	1.49×10^{-13}
GA3	-7.13	1	1.19×10^{-9}
KNO3	5.96	1	7.23×10^{-8}
COLD	0.042	1	0.96
HEAT	4.46	1	1.51×10^{-5}
LIGHT	1.54	1	0.096

Table 4.6. Example that shows how the simple effects mask the interactions. As it can be seen, GA$_3$ is always absent when there is an interaction, and vice versa.

Treatments	Soaking	NaOCl	GA$_3$	Soaking x Bleach
1	-1	-1	-1	+1
2	-1	-1	-1	+1
3	-1	+1	+1	-1
4	-1	+1	+1	-1
5	+1	-1	+1	-1
6	+1	-1	+1	-1
7	+1	+1	-1	+1
8	+1	+1	-1	+1

4. 2. Root phenotyping

4. 2. 1. Determining the optimum polyethylene glycol concentration

As can be seen in Figure 3.4, 7% of polyethylene glycol lead to excessive stress, where roots where suberized. As the aim of the experiment is to measure the length and depth of roots in stressed state, and in this over-stressed state fine roots (the ones that make up most of the root length) grow less, this is not the appropriate concentration. At a 3% concentration of polyethylene glycol, on the other side, the presence of stress is seen when comparing to the control, but is not excessive (Figure 4.4). The hydraulic conductivity was measured with an osmometer and the pressure was of -0.52 MPa.

Figure 4.4. From left to right: color scans of *S. melongena* two week old plantlets in 0 (left), 3% (center) and 7% (right) polyethylene glycol concentration gels.

4. 2. 2. Agar transparency evaluation

The most transparent agar was determined visually. Phytagel was the most transparent, Gelrite the second, and the industrial agar was quite opaque (Figure 4.5).
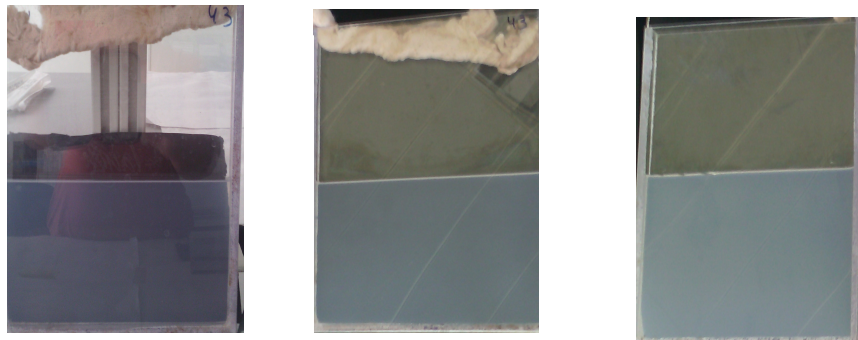

Figure 4.5: From left to right: Phytagel, Gelrite, Industrial agar

4. 2. 3. Root growth analysis

Eggplant seedlings grown in agar plates were scanned at 14 days after germination, and were analyzed using the WhinRhizo 2.3 software. The traits analyzed for the roots were: total root

length, projected area, number of tips, the thickness of the roots, and the depth to width ratio (D/W ratio). These factors helped us analyze how deeply roots grow, how much they grow and branch.

During the experiment and despite using the optimized protocol for seed germination, a lot of seeds did not germinate. Adding to the problem, the amount of seeds available was very low as they are hybrids difficult to achieve or there were few seeds remaining in the lab. Therefore for some genotypes that we included in the experiment, we have no data (*S. anguivi*, *S. lichsteinii*, *S. dasyphillum*, *S. melongena* x *S. dasyphyllum*), and for some others, we have data just for one treatment (*S. melongena* x *S. lichsteinii*, just 2 plants in the control plates; *S. melongena x S. anguivi*, just 2 plants in the water stress plates). This explains why a lot of the differences observed were statistically not significant.

The ANOVA analysis showed that there were significant differences in root length among the different genotypes when they grow in the control treatment (Table 4.7). *S. melongena* x *S. lichsteinii* hybrids outstood by their long roots (140 cm on average) followed by *S. insanum* (100 cm). *S. melongena* showed the shortest root (11 cm) and interestingly the hybrid *S. melongena* x *S. insanum* showed an intermediate length between the parents (Figure 3.6). Under PEG stress conditions *S. melongena*, *S insanum* and their hybrids did not show significant differences in root length (Figure 4.6). Therefore the effect of PEG on *S insanum* was to shorten the roots whereas in *S melongena* the effect was the contrary. *S. melongena* x *S. anguivi* showed in PEG the highest root length of the tested genotypes.

Table 4.7. One-way ANOVA results table showing the effect of the genotype within each treatment (Control and PEG).

		d.f.	Root lenght	Root length diam < 0.25 (mm)	Root length 0.25 <diam < 0.5 (mm)	Roots length diam > 0.5 (mm)	Depth to width ratio
					Mean squares		
Control	Genotype	3	10351.2 *	1351.41*	1429.93*	968.93[NS]	1.73[NS]
	Error	9	1587.09	298.37	322.72	270.55	0.47
PEG	Genotype	3	714.58[Ns]	301.99[NS]	85.20[NS]	30.49[NS]	1.78[NS]
	Error	20	305.4	114.26	80.36	49.38	0.65

[1] Degrees of freedom; [Ns], [*],[**],[***], mean non-significant, P-value < 0.05, 0.01, and 0.001 respectively
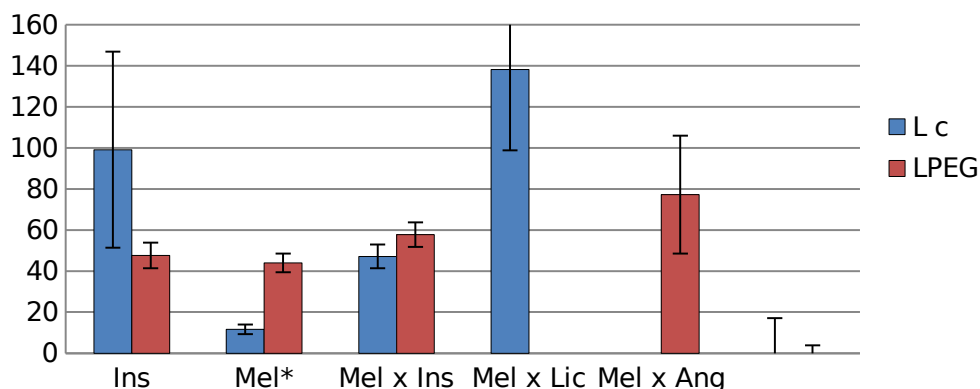
Root Lenght (cm)

Figure 4.6. Bar chart of the lengths of roots of the different genotypes in control (blue) and water stress (red) conditions. * means there were significant differences among C and PEG treatments for that genotype. Error graphs indicate standard errors.

In the analysis of the length of the roots by diameter there were again significant differences in the control treatment for root length of diam < 0.25 (mm) and root length 0.25 <diam < 0.5 (mm) (Table 4.7). There was no significant difference in the PEG treatment, though. The most common distribution of diameters in the genotypes analyzed was: to have a high length of roots of diameter between 0.25 and 0.5 mm, and then to have almost the same amount of roots (in terms of length) thinner than 0.25 mm and thicker than 0.5 mm (Figure 4.7). However in the control treatment, *S. insanum* had a higher length of roots with a diameter bellow 0.25 mm, and very few roots of a diameter higher than 0.5 mm. Interestingly this situation changed under stress condition where the *S. insamum* root diameter structure resembles more the structure of other genotypes (Figure 4.7 B). In the water stress treatment, only the *S. melongena x S. anguivi* hybrid has longer fine roots than thick ones (Figure 4.7 B). This means that *S. insanum* is able to absorb more water per total root length unit in control conditions (and the *S. melongena x S. anguivi* hybrid can do the same for water stress conditions).

The thickness of roots depends on their age. The younger roots usually have a smaller diameter than old ones, have less suberin and a thinner cuticle, and are able to absorb water and nutrients better than older, thicker ones. Small fine root diameter is associated with better plant productivity under drought conditions (Comas et al. 2013). That's why the distribution of the diameters of roots (whether there are more thin roots than thick ones) is important. The more thin roots a plant has, the more water it can absorb. Besides, as the root takes the water from the soil, the root-to-soil interface tends to accumulate salts, so growing away from this surface makes it possible to absorb more water with a smaller metabolic cost (Stirzaker and Passioura, 2006). However the changes in the distribution of diameters in our roots does not seem to follow this scheme. For example, Mel keep the same distribution but increased the length of any type of root diameter. The same happened with the *S. melongena* x *S. insanum* hybrid.
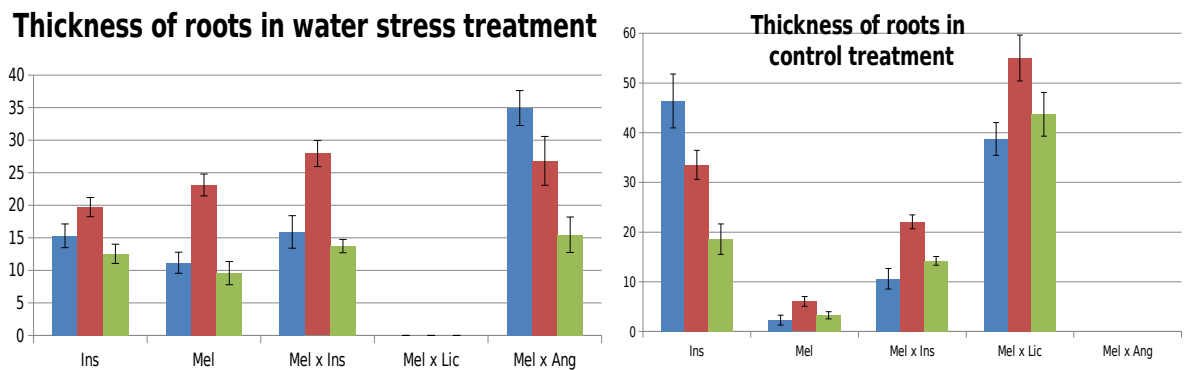
Figure 4.7. Bar chart showing the length of roots in the control (left, A) and water stress (right, B) treatment according to their thickness. The blue ones have a diameter of less than 0.25mm, the red ones have a diameter between 0.25 and 0.5 mm, and the green ones have a diameter bigger than 0.5 mm. In the left, plants from the control treatment, on the right, stressed plants. The error graphs show the square root of the standard error.
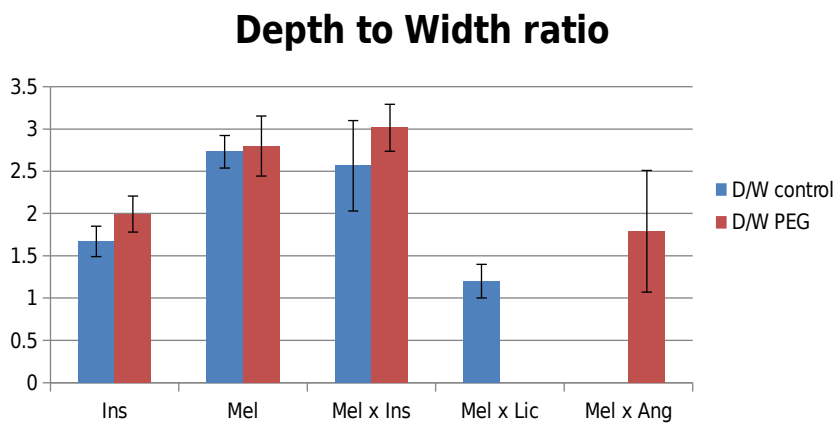

Figure 4.8 The depth to width ratio for the different genotypes. The error graphs only show difference between genotypes in each treatment. There where no significant differences within the genotypes.

The depth to width ratio shows how the roots grow: if the roots grow more to depth than to width, the D/W will be bigger. The higher root density at bigger depths is associated with water stress tolerance, as shown by White and Kirkegaard (2010). Despite there were different values of this parameter among the genotypes tested those differences were no statistically significant. And, although there is no significant difference either between D/W ratios between treatments, it can be observed in figure 4.9 that roots subjected to water stress seem to have a higher D/W ratio. This could be confirmed if there was more data. As can be seen in figure 4.10, *S. melongena* and *S. melongena x S. insanum* have the highest D/W ratios in both control and water stress conditions; this means the roots tend to grow quite deeply.

So, although *S. melongena* and the *S. melongena x S. insanum* hybrid seem to have shorter roots than the other genotypes (Figure 4.6) they are distribute deeper.

S. insanum and its hybrid have another interesting trait: they grow long, lateral roots in response to stress (Figure 4.9). This is a very interesting property that helps plants respond to stress. In cereals, the growth of nodal roots in response to stress is a very interesting property for drought tolerance (Rostamza et al., 2013).
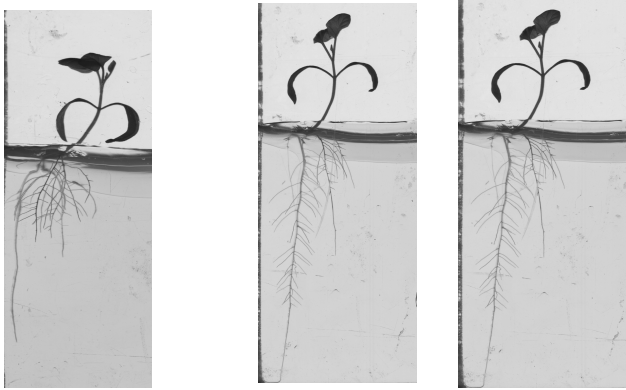

Figure 4.9. Image of *S. insanum* under stress conditions (left) and the *S. melongena x S. insanum* hybrid under stress (right)
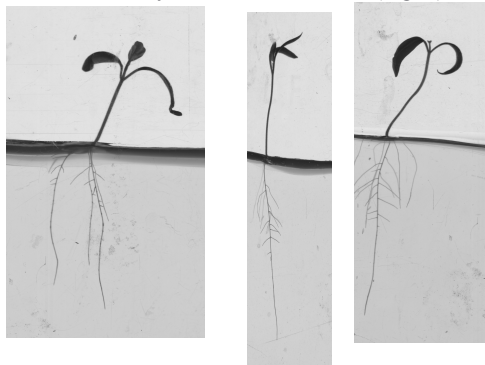

Figure 4.10. From left to right: *S. insanum*, *S. melongena*, and the *S. melongena x S. insanum* hybrid. As it can be noticed, the *S. melongena* and the hybrid root is more elongated.

4. 3. Final remarks

This work is part of a project done in Sri Lanka, Spain and the Ivory Coast with the objective to use eggplant wild relatives for improving eggplant against drought (among others). One of the partial objectives of the project was to obtain an easy and reliable protocol to phenotype water stress response in eggplant seedlings. Previous works have been done in plants grown in pots but it was decided that studying the root system and evaluating the response *in vitro* could give a lot of information.

Determining the tolerance of a plant to drought is difficult, since it is a complex, multi-genetic trait. And, although a plant's response varies wildly at different stages of development, tolerance to water scarcity is important at all stages of a plant's growth: germination, seedling, adult plant, flowering, and giving fruit. By keeping seedlings in plates subjected to water stress, drought tolerance at early stages of the plant's life can be measured.

During the experiment we experienced some problems due to the uneven and low germination of the wild eggplants and their hybrids. This problem has been persistent during all the group's work, so the trial was designed to find the factors that best help to germinate *S. melongena* seeds. Of course, it would be good to design germination protocols for the wild eggplants (*S. anguivi*, *S. lichsteinii*) also, but that was not possible due to the limited number of seeds. This trial was also done in *S. torvum*, an eggplant with lots of problems to germinate, and the results were quite good. But even so, the protocol may not be as good for other wild eggplants.

Due to the low number of plants, the results were hard to interpret, and were not significant in a lot of cases. But the tentative results obtained show that, if the problem of the germination is solved, this protocol could be quite useful to phenotype plants tolerant for drought.

5. CONCLUSION

The germination protocol for *in vitro* growth of eggplants was established successfully, and includes easy and inexpensive treatments: soaking, KNO_3 and placing the plate in the light. Bleaching could be used if necessary (as was our case), but it has a detrimental effect on seed germination at the concentration that was used (30%).

A protocol to evaluate roots for water stress by using PEG was established. Although the results obtained were not very significant due to the low number of plants evaluated, differences in root structure have been observed between *S. melongena* and its relatives (the tendency of *S. insanum* to generate adventitious roots under water stress is interesting and require further study).

The differences in root architecture that can be observed through this method of phenotyping are: total root length, root branching, the vertical distribution of the roots, and the thickness distribution of roots.

6. BIBLIOGRAPHY

ADEBOLA, P.O. and AFOLAYAN, A.J. 2006. Germination responses of Solanum aculeastrum, a medicinal species of the Eastern Cape, South Africa. *Seed Science and Technology*, vol. 34, no. 3, pp. 735–740.

ANESE, S., DA SILVA, E.A.A., DAVIDE, A.C., ROCHA FARIA, J.M., SOARES, G.C.M., MATOS, A.C.B. and TOOROP, P.E. 2011. Seed priming improves endosperm weakening, germination, and subsequent seedling development of *Solanum lycocarpum* St. Hil. *Seed Science and Technology*, vol. 39, pp. 125–139.

AROCA, R., PORCEL, R. and RUIZ-LOZANO, J.M. 2011. Regulation of root water uptake under abiotic stress conditions. *Journal of Experimental Botany*, vol. 63, no. 1, pp. 43–57.

BARRA, M., CORREA, J., SALAZAR, E. and SAGREDO, B. 2013. Response of Potato (Solanum tuberosum L.) Germplasm to Water Stress Under In Vitro Conditions. *American Journal of Potato Research*, vol. 90, no. 6, pp. 591–606.

BATAK, I., DEVIĆ, M., GIBA, Z., GRUBISIĆ, A., POFF, K.L. and KONJEVIĆ, R. 2002. The effects of potassium nitrate and NO-donors on phytochrome A- and phytochrome B-specific induced germination of Arabidopsis thaliana seeds. *Seed Science Research*, vol. 12, no. 4, pp. 253–259.

BEHBOUDIAN, M.H. 1977. Responses of eggplant to drought. I. Plant water balance. *Scientia Horticulturae*, vol. 7, no. 4, pp. 303–310.

BEN-GAL, A., YERMIYAHU, U. and COHEN, S. 2009. Fertilization and Blending Alternatives for Irrigation with Desalinated Water. *Journal of Environment Quality*, vol. 38, no. 2.

BENGOUGH, A.G. 2005. Root responses to soil physical conditions; growth dynamics from field to cell. *Journal of Experimental Botany*, vol. 57, no. 2, pp. 437–447.

BLUM, A. 2011. *Plant breeding for water-limited environments*. New York: Springer Science+Business Media.

BOURZAC, K. 2013. Water: The flow of technology. *Nature*, vol. 501, no. 7468, pp. 4–6.

BUKENYA, Z. R., AND CARASCO, J. F. (1994). Biosystematic study of Solanum macrocarpon-S. dasyphyllum complex in Uganda and relations with S. linnaeanum. East Afr. Agric. Fores. J. 59, 187–204.

BUREAU, U.C. [Consulted: 19 June 2015]. Available at: http://www.census.gov/.

COMAS, L.H., BECKER, S.R., CRUZ, V.M.V., BYRNE, P.F. and DIERIG, D.A. 2013. Root traits contributing to plant productivity under drought. *Frontiers in Plant Science*, vol. 4.

COURTOIS, B., CHAITEP, W., MOOLSRI, S., SINHA, P.K., TREBUIL, G., YADAV, R., 1996. Drought resistance and germplasm improvement. Ongoing research in the upland rice research consortium, in: Piggin, C., Courtois, B., Schmit, V. (Eds.), Upland rice research in partnership. IRRI Discussion Paper Series 16, 154–175.

COURTOIS, B., MCLAREN, G., SINHA, P.K., PRASAD, K., YADAV, R., SHEN, L., 2000. Mapping QTLs associated with drought avoidance in upland rice. Mol. Breeding 6 (1), 55–66.

DAUNAY M.C., LESTER R.N., ANO G. (2001) Eggplant. Tropical plant breeding. Montpellier: Science Publishers, 199–222.

DEL MONTE, J.P., AGUADO, P.L. and TARQUIS, A.M. 2014. Thermal time model of Solanum sarrachoides germination. *Seed Science Research*, vol. 24, no. 04, pp. 321–330.

DILLEHAY, T., KOLATA, A.L. and PINO, M. 2004. Pre-industrial human and environment interactions in northern Peru during the late Holocene. *The Holocene*, vol. 14, no. 2, pp. 272–281.

DOWNIE, H., HOLDEN, N., OTTEN, W., SPIERS, A.J., VALENTINE, T.A. and DUPUY, L.X. 2012. Transparent Soil for Imaging the Rhizosphere. *PLoS ONE*, vol. 7, no. 9.

EAST, R. 2013. Microbiome: Soil science comes to life. *Nature*, vol. 501, no. 7468, pp. 18–19.

FIORUCCI F. 2014. Tolleranza allo stress idrico in genotipi di melanzana e specie affini. Master thesis, Università degli Studi della Tuscia .

FITA, A., PICÓ, B., MONFORTE, A.J., NUEZ, F. and FITA, A. 2008. Genetics of Root System Architecture Using Near-isogenic Lines of Melon. *Journal of the American Society for Horticultural Science*, vol. 133, pp. 448–458.

FURINI, A. and WUNDER, J. 2004. Analysis of eggplant (Solanum melongena)-related germplasm: morphological and AFLP data contribute to phylogenetic interpretations and germplasm utilization. *Theoretical and Applied Genetics*, vol. 108, no. 2, pp. 197–208.

GISBERT, C., PROHENS, J., RAIGÓN, M.D., STOMMEL, J.R. and NUEZ, F. 2011. Eggplant relatives as sources of variation for developing new rootstocks: Effects of grafting on eggplant yield and fruit apparent quality and composition. *Scientia Horticulturae*, vol. 128, no. 1, pp. 14–22.

HENRY, A., GOWDA, V.R.P., TORRES, R.O., MCNALLY, K.L. and SERRAJ, R. 2011. Variation in root system architecture and drought response in rice (Oryza sativa): Phenotyping of the OryzaSNP panel in rainfed lowland fields. *Field Crops Research*, vol. 120, no. 2, pp. 205–214.

HURTADO, M., VILANOVA, S., PLAZAS, M., GRAMAZIO, P., FONSEKA, H.H., FONSEKA, R. and PROHENS, J. 2012. Diversity and Relationships of Eggplants from Three Geographically Distant Secondary Centers of Diversity. *PLoS ONE*, vol. 7, no. 7.

KANDARI, L.S., KULKARNI, M.G. and VAN STADEN, J. 2011. Effect of Nutrients and Smoke Solutions on Seed Germination and Seedling Growth of Tropical Soda Apple (Solanum viarum). *Weed Science*, vol. 59, no. 4, pp. 470–475.

KARAM, F., SALIBA, R., SKAF, S., BREIDY, J., ROUPHAEL, Y. and BALENDONCK, J. 2011. Yield and water use of eggplants (Solanum melongena L.) under full and deficit irrigation regimes. *Agricultural Water Management*, vol. 98, no. 8, pp. 1307–1316.

KNAPP, S., VORONTSOVA, M.S. and PROHENS, J. 2013. Wild Relatives of the Eggplant (Solanum melongena L.: Solanaceae): New Understanding of Species Names in a Complex Group. *PLoS ONE*, vol. 8, no. 2.

KOO, H.J., PARK, S.M., KIM, K.P., SUH, M.C., LEE, M.O., LEE, S.-K., XINLI, X. and HONG, C.B. 2015. Small Heat Shock Proteins Can Release Light Dependence of Tobacco Seed during Germination. *Plant Physiology*, vol. 167, no. 3, pp. 1030–1038.

LESTER, R.N. and HASAN, S.M.Z. 1990. The Distinction between Solanum incanum L. and Solanum insanum L. (Solanaceae). *Taxon*, vol. 39, no. 3.

LESTER, R. N., JAEGER, P. M. L., BLEIJENDAAL-SPIERINGS, B. H. M., BLEIJENDAAL, H. P. O. and HOLLOWAY, H. L. O. (1990). African eggplants – a review of collecting in West Africa. FAO/IBPGR Plant Genet. Resour. Newsl. 81–82, 17–26.

LESTER, R. N., and NIAKAN, L. (1986). Origin and domestication of the scarlet eggplant, Solanum aethiopicum, from S. anguivi in Africa, in Solanaceae: Biology and Systematics, (New York, NY: Columbia University Press), 433–456.

LESTER, R. N. and THITAI, G. N. W. (1989). Inheritance in Solanum aethiopicum, the scarlet eggplant. Euphytica 40, 67–74.

LITTLE, T. HILLS, J. 1978. Agricultural experimentation: design and analysis. Wiley, New York.

LYNCH, J.P. 2007. TURNER REVIEW No. 14. Roots of the Second Green Revolution. *Australian Journal of Botany*, vol. 55, no. 5, pp. 493–512.

MACE, E., LESTER, R. and GEBHARDT, C. 1999. AFLP analysis of genetic relationships among the cultivated eggplant, Solanum melongena L., and wild relatives (Solanaceae). *Theoretical and applied genetics*, no. 99, pp. 626–633.

MAROTO, J.V., 1992. *Horticultura herbácea especial*. 1992. 3. Madrid: Ediciones Mundi-Prensa.

MASEDA, P.H. and FERNANDEZ, R.J. 2006. Stay wet or else: three ways in which plants can adjust hydraulically to their environment. *Journal of Experimental Botany*, vol. 57, no. 15, pp. 3963–3977.

MATHIEU, L., LOBET, G., TOCQUIN, P. and PÉRILLEUX, C. 2015. 'Rhizoponics': a novel hydroponic rhizotron for root system analyses on mature Arabidopsis thaliana plants. *Plant Methods*, vol. 11.

MAUNDU, P., ACHIGAN-DAKO, E. and MORIMOTO, Y. (2009). Biodiversity of African Vegetables, African Indigenous Vegetables in Urban Agriculture, London: Earthscan, 65–104.

MEISTER, R., RAJANI, M.S., RUZICKA, D. and SCHACHTMAN, D.P. 2014. Challenges of modifying root traits in crops for agriculture. *Trends in plant science*, vol. 19, pp. 779–788.

O'SHAUGHNESSY, S.A., EVETT, S.R., COLAIZZI, P.D. and HOWELL, T.A. 2012. A crop water stress index and time threshold for automatic irrigation scheduling of grain sorghum. *Agricultural Water Management*, vol. 107, pp. 122–132.

PANDRANGI, S., ELWELL, M.W., ANANTHESWARAN, R.C. and LABORDE, L.F. 2006. Efficacy of Sulfuric Acid Scarification and Disinfectant Treatments in Eliminating Escherichia coli O157: H7 from Alfalfa Seeds Prior to Sprouting. *Journal of Food Science*, vol. 68, no. 2, pp. 613–617.

PASSAM, H.C., THEODOROPOULOU, S., KARANISSA, T. and KARAPANOS, I.C. 2010. Influence of harvest time and after-ripening on the seed quality of eggplant. *Scientia Horticulturae*, vol. 125, no. 3, pp. 518–520.

PENELLA, C., NEBAUER, S.G., BAUTISTA, A.S., LÓPEZ-GALARZA, S. and CALATAYUD, Á. 2014. Rootstock alleviates PEG-induced water stress in grafted pepper seedlings: Physiological responses. *Journal of Plant Physiology*, vol. 171, no. 10, pp. 842–851.

PIONEER.[Consulted: 5 July 2015]. Available at: http://www.pioneer.com.

PITERKOVA, J., LUHOVA, L., HOFMAN, J., TURECKOVA, V., NOVAK, O., PETRIVALSKY, M. and FELLNER, M. 2012. Nitric oxide is involved in light-specific responses of tomato during germination under normal and osmotic stress conditions. *Annals of Botany*, vol. 110, no. 4, pp. 767–776.

PLAZAS, M., ANDÚJAR, I., VILANOVA, S., GRAMAZIO, P., HERRAIZ, J.F. and PROHENS, J. 2014. Conventional and phenomics characterization provides insight into the diversity and relationships of hypervariable scarlet (Solanum aethiopicum L.) and gboma (S. macrocarpon L.) eggplant complexes. *Frontiers in Plant Science*, vol. 5, no. 318, pp. 1–13.

PRABHAVATHI, V., YADAV, J.S., KUMAR, P.A. and RAJAM, M.V. 2002. Abiotic stress tolerance in transgenic eggplant (Solanum melongena L.) by introduction of bacterial mannitol phosphodehydrogenase gene. *Molecular Breeding*, vol. 9, no. 2, pp. 137–147.

PRICE, A.H., TOWNSEND, J., JONES, M.P., AUDEBERT, A. and COURTOIS, B. 2002. Mapping QTLs associated with drought avoidance in upland rice grown in the Philippines and West Africa. *Plant Molecular Biology*, vol. 48, no. 5/6, pp. 683–695.

RIBAUT, J.M. and RAGOT, M. 2006. Marker-assisted selection to improve drought adaptation in maize: the backcross approach, perspectives, limitations, and alternatives. *Journal of Experimental Botany*, vol. 58, no. 2, pp. 351–360.

ROSTAMZA, M., RICHARDS, R.A. and WATT, M. 2013. Response of millet and sorghum to a varying water supply around the primary and nodal roots. *Annals of Botany*, vol. 112, no. 2, pp. 439–446.

ROY, R.K. 2011. A primer of the Taguchi method. Society of Manufacturing Engineers, Dearborn, Michigan, USA.

SALEEM, M.S., SAJID, M., AHMED, Z., AHMED, S. and AHMED, N. 2014. Effect of Seed Soaking On Seed Germination and Growth of Bitter Gourd Cultivars. *Journal of Agriculture and Veterinary Science*, vol. 6, no. 6, pp. 7–11.

SCHULZ, H., POSTMA, J.A., VAN DUSSCHOTEN, D., SCHARR, H. and BEHNKE, S. 2013. Plant Root System Analysis from MRI Images. *Communications in Computer and Information Science*, pp. 411–425.

SCHULZ, H., POSTMA, J.A., VAN DUSSCHOTEN, D., SCHARR, H. and BEHNKE, S. 2013. Plant Root System Analysis from MRI Images. *Communications in Computer and Information Science*, pp. 411–425.

SEGAL, E., BEN-GAL, A. and SHANI, U. 2006. Root Water Uptake Efficiency Under Ultra-High Irrigation Frequency. *Plant and Soil*, vol. 282, no. 1-2, pp. 333–341. ISSN 1573-5036. DOI 10.1007/s11104-006-0003-6.

SHALIMU, D., QIU, J., TAN, D., BASKIN, C.C. and BASKIN, J.M. 2012. Seed Biology of the Invasive Species Buffalobur (Solanum rostratum) in Northwest China. *Weed Science*, vol. 60, no. 2, pp. 219–224.

SHI, S., FAN, M., IWAMA, K., LI, F., ZHANG, Z. and JIA, L. 2015. Physiological basis of drought tolerance in potato grown under long-term water deficiency. *International Journal of Plant Production*, vol. 9, pp. 305–320.

STEELE, K., 2009. Novel upland rice variety bred using marker-assisted selection and client-oriented breeding released in Jharkhand. Bangor University, Bangor, India.

STEELE, K.A., PRICE, A.H., SHASHIDHAR, H.E. and WITCOMBE, J.R. 2005. Marker-assisted selection to introgress rice QTLs controlling root traits into an Indian upland rice variety. *Theoretical and Applied Genetics*, vol. 112, no. 2, pp. 208–221.

STEELE, K.A., VIRK, D.S., KUMAR, R., PRASAD, S.C. and WITCOMBE, J.R. 2007. Field evaluation of upland rice lines selected for QTLs controlling root traits. *Field Crops Research*, vol. 101, no. 2, pp. 180–186. DOI 10.1016/j.fcr.2006.11.002.

STIRZAKER, R.J. and PASSIOURA, J.B. 1996. The water relations of the root-soil interface. *Plant, Cell and Environment*, vol. 19, no. 2, pp. 201–208.

SUDARMONOWATI, E., HARTATI, N.S. and KURNIAWATI, S. 2012. Drought resistant eggplant selection confirmed by genetic marker. *Proc Soc Indon Biodiv Intl Conf*, vol. 1, pp. 64–69.

SYNGENTA GLOBAL WEBSITE – Bringing plant potential to life. [Consulted: 5 July 2015]. Available at: http://www3.syngenta.com.

TAAB, A. and ANDERSSON, L. 2009. Seasonal changes in seed dormancy of Solanum nigrum and Solanum physalifolium. *Weed Research*, vol. 49, no. 1, pp. 90–97.

TIMMERMANN, A., OBERHUBER, J., BACHER, A., ESCH, M., LATIF, M. and ROECKNER, E. 1999. Increased El Niño frequency in a climate model forced by future greenhouse warming. *Nature*, vol. 398, no. 6729, pp. 694–697.

VANDOORNE, B., MATHIEU, A.-S., VAN DEN ENDE, W., VERGAUWEN, R., PERILLEUX, C., JAVAUX, M. and LUTTS, S. 2012. Water stress drastically reduces root growth and inulin yield in Cichorium intybus (var. sativum) independently of photosynthesis. *Journal of Experimental Botany*, vol. 63, no. 12, pp. 4359–4373.

WAN, C., XU, W., SOSEBEE, R.E., MACHADO, S. and ARCHER, T. 2000. Hydraulic lift in drought-tolerant and -susceptible maize hybrids. *Plant and Soil*, vol. 219, no. 1/2, pp. 117–126.

WEESE, T.L. and BOHS, L. 2010. Eggplant origins: Out of Africa, into the Orient. *Taxon*, vol. 59, pp. 49–56.

WEI, S., ZHANG, C., CHEN, X., LI, X., SUI, B., HUANG, H., CUI, H., LIU, Y., ZHANG, M. and GUO, F. 2010. Rapid and Effective Methods for Breaking Seed Dormancy in Buffalobur (Solanum rostratum). *Weed Science*, vol. 58, no. 2, pp. 141–146.

WHITE, R.G. and KIRKEGAARD, J.A. 2010. The distribution and abundance of wheat roots in a dense, structured subsoil--implications for water uptake. *Plant, Cell & Environment*, vol. 33, no. 2, pp. 133–148.

YERMIYAHU, U., TAL, A., BEN-GAL, A., BAR-TAL, A., TARCHITZKY, J. and LAHAV, O. 2007. ENVIRONMENTAL SCIENCE: Rethinking Desalinated Water Quality and Agriculture. *Science*, vol. 318, no. 5852, pp. 920–921.

YOGEESHA, H.S., UPRETI, K.K., PADMINI, K., BHANUPRAKASH, K. and MURTI, G.S.R. 2006. Mechanism of seed dormancy in eggplant (*Solanum melongena* L.). *Seed Science and Technology*, vol. 34, pp. 319–325.

ZHOU, B., LU, N., WANG, Z. and YA, X. 2010. Effect of Grafting to Eggplant Growth and Resistance Physiology under NaCl. *China Vegetables*,

ZHU, M., BROWN, K. and LYNCH, J. 2010. Root cortical aerenchyma improves the drought tolerance of maize (Zea mays L.). *Plant, Cell & Environment*, vol. 33, pp. 740–749.