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Title:

Sources of parthenocarpy for Zucchini breeding: relationship with ethylene production and sensitivity

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Abstract

Parthenocarpy is becoming an essential trait for off-season greenhouse production of Zucchini squash. Given that winter conditions promote a reduction in the number of male flowers and in the activity of pollinators, the application of synthetic auxins is currently the most widespread method to induce fruit set. We have explored landraces with elongated fruits from the morphotypes Zucchini, Vegetable Marrow and Cocozelle from *C. pepo*, to identify new sources of parthenocarpy for Zucchini breeding programs. From a first screening with 45 landraces and 3 hybrids that are commercialized as parthenocarpic, genotypes were selected for their capacity to produce parthenocarpic fruits of marketable size. Eleven landraces, which received similar scores to those of the control hybrids, were selected as parthenocarpic for further analysis. In the subsequent screening, the growth rate of unpollinated fruits was studied in selected parthenocarpic and non-parthenocarpic cultivars from the first screening. Besides the three control hybrids, the fastest parthenocarpic fruit growth was detected in landraces *CpCAL112*, *CM-37*, *E-27*, *PI261610*, and *V-185*. The source of the parthenocarpy of some of these landraces differs from that of the hybrids, since it was not found to be associated with the conversion of female into bisexual flowers or with the so-called syndrome of fruits with attached flowers, an undesirable associated trait in current parthenocarpic hybrids. Moreover, we have demonstrated that the parthenocarpy of these landraces is correlated with a downregulation of ethylene production in the unpollinated fruits during the days immediately after anthesis. In the non-parthenocarpic cultivars unpollinated fruits produced a boost of ethylene at 3 DPA, concomitantly with fruit abortion and senescence, while in parthenocarpic ones the fruits barely produced ethylene at 3DPA. Therefore, ethylene production in ovary/fruits at 3 DPA could be used as a marker to identify and select parthenocarpy in Zucchini squash. Nevertheless, earlier evaluations of ethylene production and sensitivity in vegetative organs and in male flowers are not so well correlated with parthenocarpy in the analyzed cultivars.

Keywords: *Cucurbita pepo*, parthenocarpy, andromonoecy, ethylene production, ethylene sensitivity

Introduction

The genus *Cucurbita* is made up of 25 wild and cultivated species and shows the largest morphological diversity of the *Cucurbitaceae* family (Decker, 1988). *C. pepo* is one of the three most important species of the genus as regards both its worldwide distribution and its economic value (Nee, 1990). Based on different molecular studies, the species has been divided into two subspecies: *C. pepo* ssp. *pepo* and *C. pepo* ssp. *ovifera* (Decker, 1988; Wilson et al, 1992; Jobts et al, 1998; Katzir et al, 2000). Moreover, the variability in size, shape, color and texture of fruits has led to the classification of *C. pepo* into eight different botanical morphotypes: Scallop, Acorn, Crookneck and Straightneck are included in the spp. *ovifera*, and Pumpkin, Vegetable Marrow, Zucchini and Cocozelle in the ssp. *pepo* (Paris, 1989, Paris et al, 2003; Paris and Janick, 2005). Within the subspecies *pepo*, Zucchini, Vegetable Marrow and Cocozelle are grouped together, separate from Pumpkin, based on both morphological and molecular markers (Paris et al, 2003; Ferriol et al, 2003).

C. pepo is a monoecious crop with two or three sexual phases of development. In the first phase only male flowers are produced. This is followed by a phase during which male and female flowers alternate, and the final phase consist only of female flowers (Peñaranda et al, 2007; Manzano et al, 2010; 2013). This latter phase restricts pollination and fruit set, but only occurs in certain cultivars when grown under winter conditions (Peñaranda et al, 2007). Since female and male sexual organs are separated in unisexual flowers, fruit set and development in squash requires the activity of pollinators, a process that is not only restricted to the six hours that male and female flowers remain open during the morning (Nepi and Pacini, 1993), but which is also highly dependent on environmental conditions that affect both the number of female and male flowers per plant and pollinator activity (Wien et al, 2002; Loy, 2012). In off-season greenhouse productions, i.e. under conditions in which plants produce a reduced number of male flowers and the activity of pollinators is very limited, natural pollination is difficult, and fruit set is currently induced by synthetic auxins (Sanz, 1995; Wien, 2002). Nevertheless, the demand for healthier and environmentally friendly fresh products is making parthenocarpy a priority trait in current breeding programs of Zucchini.

Among cucurbits, cucumber was the first species in which the introduction of parthenocarpic cultivars improved off-season production in greenhouses (Sturtevant, 1890), but parthenocarpy has been detected in other species of the family, including melon and squash (Rylski and Aloni, 1990). The earliest studies in the parthenocarpy of *C. pepo* were made by Durtham (1925), who closed 301 female flowers to prevent pollination, and found no parthenocarpic production in squash. The first squash variety described as parthenocarpic was *Royal Acorn* (Nitsch, 1952), although its parthenocarpic potential was later questioned (Robinson and Reiners, 1999). Subsequent studies identified a number of cultivars with parthenocarpic potential, including *Dg-4* and *Poseidon* (Nijs and Balder, 1983); the cultivar *Chefini* (Robinson, 1993), and *Whitaker*, whose parthenocarpy appears to be controlled by one major dominant gene (De Menezes et al, 2005). Nijs and Zanten (1982), Om and Hong (1989) and Robinson and Reiners (1999) studied the parthenocarpic potential of different *C. pepo* cultivars, concluding that the highest level of parthenocarpy is found in genotypes with dark green skin from the morphotype Zucchini and Cocozelle. It appears, therefore, that cultivars with elongated fruits exhibit the highest parthenocarpic fruit production. Nevertheless, the production of parthenocarpic fruits in a given cultivar is quite dependent on environmental conditions. It is known that winter conditions, especially low temperatures, are able to promote parthenocarpy in *C. pepo* (Globerson, 1971; Rylski, 1974b; Nijs and Balder, 1983; Rylski and Aloni, 1990; Robinson and Reiners, 1993; Gómez et al, 2004).

Despite the above studies, to date few Zucchini squash cultivars are marketed as parthenocarpic. The previously identified parthenocarpic cultivars, such as *Whitaker* and *Chefini*, showed no parthenocarpic potential in greenhouse production in the southeast of Spain (unpublished data). Under these specific conditions, we found only three hybrids that are commercialized as parthenocarpic: *Cavili*, *Parthenon* and *Argo*. These hybrids have been subjected to different evaluations by our research group since 2004, and they all produce parthenocarpic fruits with attached flowers, an undesirable associated trait that results from the delay in maturation and abscission of floral organs (Peñaranda et al, 2007; Martínez et al, 2013). As the fruit is not commercialized with

the flower, its manual abscission causes a wound which leads to rapid loss of water and decay during postharvest storage. In *Cavili*, parthenocarpic fruits have been found to be derived not from female flowers but from bisexual ones, which are induced in these cultivars when greenhouse temperature rises above 30ºC (Martínez et al, 2013). This type of parthenocarpy has no value for Zucchini greenhouse production, as the temperature easily exceeds 30 °C not only during spring and summer, but also on occasions in the autumn-winter season. Given these considerations, it would therefore be necessary to identify new sources of parthenocarpy for Zucchini greenhouse breeding programs.

Fruit set and subsequent fruit growth is a vital phase of plant development that is largely dependent on the biosynthesis and crosstalk of phytohormones, such as auxins, GAs and cytokinins, produced in the pollinated ovaries (Ozga and Reinecke, 2003; Srivastava and Handa, 2005; De Jong et al, 2009). To date, however, the involvement of other hormones such as ethylene in this developmental process has not been studied in depth. Our research group has recently found that fruit set and early fruit development of Zucchini after pollination and fertilization requires a low level of ethylene in the pollinated ovaries for a few days after anthesis, and that the lack of pollination induces a boost of ethylene in the fruit 3 days after anthesis, which is concomitant with fruit abortion and senescence (Martínez et al, 2013). Therefore, although the parthenocarpic development of the fruit is normally achieved by the coordinated action of GAs, auxins and cytokinins (Srivastava and Handa, 2005; Serrani et al, 2010), we have recently demonstrated that the parthenocarpy of the Zucchini cultivar *Cavili* is also associated with a low level of ethylene in the unpollinated fruit for a few days after anthesis (Martínez et al, 2013). This reduction in ethylene production could be responsible for the conversion of female into bisexual flowers and the delay in the maturation and abscission of floral organs that is normally associated with the parthenocarpy of this cultivar (Payán et al, 2006; Peñaranda et al, 2007; Martínez et al, 2013).

In this paper we have not only sought new sources of parthenocarpy in squash, but also determined the correlation between parthenocarpy and ethylene production and

sensitivity. Our results indicate the existence of a negative correlation in squash between the production of ethylene in the unpollinated ovaries at 3 DPA and the parthenocarpic development of fruit. Therefore, the production of ethylene in unpollinated fruits in the few days after anthesis could be used as a physiological marker for the identification and selection of parthenocarpy in this vegetable crop.

Materials and Methods

Plant material and culture conditions

The present study used forty-five cultivars of *Cucurbita pepo* from the core collection of the germplasm bank at the Polytechnic University of Valencia (COMAV), as well as certain cultivars from the seed bank of the University of Almería (BSUAL) (Table 1). All produce fruits of elongated phenotype, belonging to types Zucchini, Vegetable Marrow and Cocozelle of *C. pepo* spp. *pepo*. The evaluated germplasm represents Spanish landraces from different regions of the Iberian peninsula, mainly Valencia and Andalucía, but also from the Canary Islands. However, four of the cultivars originated in Greece, Yugoslavia, Morocco and Turkey (Table 1). The hybrids *Argo*, *Cavili* and *Parthenon* were used as positive controls of parthenocarpy.

Evaluations were made in autumn-winter seasons 2009-10 and spring 2011. On both occasions, seeds from each cultivar were germinated on nursery trays under high humidity conditions for a period of 15 days, and then transplanted to soil. Plants were grown in a greenhouse in Almeria (Spain) following standard local commercial practices for both plant nutrition and pest and disease control.

Phenotypic evaluation of cultivars

In 2009-2010 we screened 45 traditional cultivars belonging to the morphotypes Zucchini, Vegetable Marrow and Cocozelle (Table 1). 10 plants of each accession were classified according to the fruit morphotype, and evaluated for their habit of growth (bushy or vine), monoecy stability (monoecious or partially andromonoecious), and for

the level of parthenocarpy. From this first evaluation, we selected a number of cultivars that were again evaluated in 2011 for parthenocarpy, monoecy, stability and ethylene production and sensitivity. Those accessions that segregated for fruit shape and that appear include different morphotypes (Table 1), were separately multiplied for future evaluations.

We have previously observed that under high temperature conditions, certain cultivars of *C. pepo* show unstable monoecy (or partial andromonoecy), characterized by the partial conversion of female into bisexual flowers with differing degrees of stamen development (Martínez et al, in press). To evaluate monoecy instability, pistillate flowers were scored from 0 to 3 according to their degree of stamen development. Female flowers with no stamen development were scored as 0, flowers with primordial stamens as 1, those with medium-sized stamens and anthers as 2, and bisexual flowers with complete stamens and anthers able to produce pollen were scored as 3. On the basis of these bisexuality scores, we defined an andromonoecy index (AI) for each cultivar, calculated as the average bisexuality score from at least 10 plants with a minimum of 5 pistillate flowers evaluated per plant. Plants and genotypes with an AI of 0 to 0.9 were considered to be monoecious, while those with scores of 1 to 3 were considered unstable for monoecy or partially andromonoecious (Martínez et al, in press).

To evaluate parthenocarpy, plants were grown in a greenhouse free of pollinators and other insects, which ensured that all the fruits were parthenocarpic. In the first trial, conducted in autumn-winter 2009-2010 with 10 plants of each accession, cultivars were scored from 0 to 5, according to the number of marketable parthenocarpic fruits produced in 10 days. The score of each cultivar was the mean of three evaluations, and scoring was as follows: at least 10 marketable fruits scored 5, 8 to 10 fruits scored 4, 5 to 7 fruits 3, 2 to 4 fruits scored 2, 1 fruit scored 1 and no fruits scored 0. As a result of this first trial, different cultivars were selected and reassessed in 2011. In this second trial, parthenocarpy was evaluated by measuring the length and width of at least 10 parthenocarpic fruits of each cultivar over a total of 7 days post-anthesis (DPA). Measurements were taken at anthesis and 3, 5 and 7 DPA. For fruits whose growth aborted before 7 days, the last measurement before abortion was considered in the subsequent days. The findings allowed us to determine the mean fruit growth rate in

each cultivar, and to compare the increase in the fruit length and width between anthesis and each of the control points (3, 5 and 7 DPA) for each cultivar.

To assess the ethylene sensitivity of each cultivar, 10 male flower buds (1-2 days before anthesis) were removed from the plant and placed in glass jars with water in a hermetic 50 L container. The floral buds were incubated in 20 ppm of ethylene for 2 days, and the number of hours until abscission was scored in each flower up to a total of 3 days post-treatment. As a control, the same number of male flowers were treated with air and maintained in the same conditions. The essay was performed twice, and ethylene sensitivity was measured as the percentage of reduction in abscission time caused by the ethylene treatment.

Ethylene production in vegetative organs was determined in 3 replicates per sample, each containing at least 10 young leaves of 2-3 cm length. Leaves were excised from the plant and incubated at room temperature for 24 h in sealed containers in the dark for the accumulation of ethylene. On the other hand, ethylene production in fruits at 3 DPA was also measured in 3 replicates per cultivar, each containing three 3 fruits. In this case 3 fruits at the same developmental stage were harvested and enclosed in sealed containers for 6 h. Ethylene production was determined in each sample 3 times in a Varian 3900 gas chromatograph apparatus fitted with a flame ionization detector (FID), previously calibrated to determine ethylene production.

Statistical analysis

In order to determine differences in fruit development and ethylene production and sensitivity among cultivars, mean and standard error and standard deviations were calculated. Dependence between variables was studied using regression analysis. The results were evaluated fitting a linear model. The ANOVA table tells us whether or not there is a relationship between variables: F statistic tests the null hypothesis, i.e. that the slope of the regression line is equal to 0 and there is no relation between variables. R value indicates the strength of the relationship among variables, while model p-value establishes the significance level of the analysis. All analysis were performed with Statgraphics Plus v 5.1 software.

Results

Screening for parthenocarpy in *C. pepo*

With the aim of identifying new sources of parthenocarpy for Zucchini breeding programs, in this work we have evaluated the parthenocarpic potential of 45 landraces of *C. pepo* ssp. *pepo*, including 39 from the core collection in the COMAV germplasm bank at the Polytechnic University of Valencia (Ferriol et al, 2003; Nuez et al, 2000), and 6 from the seed bank at the University of Almería (BSUAL), all collected from different regions in Spain, but also from Turkey, Yugoslavia, Greece and the north of Africa (Table 1). Given that Zucchini cultivars had been reported to have the highest parthenocarpic potential, most of the cultivars evaluated were from the morphotype Zucchini, but cultivars with elongated fruits from the morphotypes Vegetable Marrow and Cocozelle were also included (Table 1). Three Zucchini hybrid cultivars, *Argo*, *Cavili* and *Parthenon*, which are commercialized as parthenocarpic were used as positive controls (Table 1).

The first assessment was conducted in autumn-winter of 2009-2010, under conditions of low temperature and short photoperiod, i.e. conditions that favor the growth of parthenocarpic fruits. From each cultivar 10 plants were grown in the absence of pollinators and hormonal treatments, and the parthenocarpic fruits that reached marketable size (16 cm in length) in a total of 10 days were recorded. The test was repeated three times and the parthenocarpy level of each variety was scored on a scale of 1 to 5, depending on the average production of marketable parthenocarpic fruits. Cultivars producing 10 or more such fruits were scored as 5, while those producing fewer than 2 marketable fruits during the same period were scored as 1 (see Materials and Methods). Three of the commercial hybrids (*Argo*, *Cavili* and *Parthenon*) and 2 traditional cultivars (*CpCAL112* and *CpCAL110*) belonging to the morphotype Zucchini received scores of over 4 (Table 1). A total of 11 cultivars that obtained a mean parthenocarpic of over 2.75 were selected for further analysis. Another 6 cultivars with a mean score of less than 1.50 were also selected as contrasting materials.

The *C. pepo* cultivars were also classified according to their growth habit (vine or bushy type), sex expression (monoecious or partially andromonoecious), and fruit morphology (Zucchini, Vegetable Marrow or Cocozelle) (Table 1). In certain cultivars some of these traits were still segregating, producing plants whose fruits were classified in different morphotypes. In accordance with visual observations and previous evaluations (Ferriol et al, 2003), *AFR-12*, *A-17*, *C-1*, *C-9*, *V-142*, *V-171*, *V-32* were classified as both Zucchini and Vegetable Marrow; *V-116* and *V-185* produced fruits of the morphotypes Zucchini and Cocozelle; while cultivars *CA-154* and *CpCAL097* produced fruits of morphotypes Cocozelle and Vegetable Marrow (Table 1). In these cases, plants from different morphotypes were reproduced separately, and the offspring from plants belonging to the morphotype with the higher production of parthenocarpic fruits, normally Zucchini, were used for the second evaluation.

The second screening was conducted with 20 cultivars selected from the first essay. This study was performed in the spring season of 2011 in environmental conditions that did not favor the parthenocarpic growth of the fruit and where it could be easier, therefore, to discriminate between parthenocarpic and non-parthenocarpic cultivars. Since the number of varieties tested was lower, we studied the longitudinal and diametrical growth rate of at least 10 unpollinated fruits of each cultivar from anthesis up to 7 days post-anthesis (DPA), when a pollinated fruit has already reached marketable size. The length and caliber of the fruits were measured at 0, 3, 5 and 7 DPA. The longitudinal fruit growth patterns in the different cultivars are shown in Figure 1. The relative growth rates of fruit caliber were similar to those of fruit length (data not shown). In many of the cultivars that were classified as non-parthenocarpic, the unpollinated fruit aborted after 3DPA, while in others the fruit growth rate was so low that they did not reach marketable size at 7DPA (Fig. 1). Nevertheless, in those cultivars that were classified as parthenocarpic, the growth of the fruit increased progressively throughout the 7 days of study, reaching marketable length before or at 7 DPA (Fig. 1). The evaluation of parthenocarpy by this assessment method is much more accurate than that used in the first trial. In fact, in six of the eleven cultivars that were initially selected as parthenocarpic, *CpCAL110*, *AFR-12*, *PI169462*, *Pascual 40*, *Greece 6* and *CpCAL044*, the potential parthenocarpic growth of their fruits proved insufficient to reach marketable size at 7 DPA (Fig. 1). These cultivars were finally classified as non-parthenocarpic (Fig. 1).

Although the most parthenocarpic cultivars already showed a higher ovary size at anthesis (Fig. 1), marketable fruit size was reached in the cultivars with the highest fruit growth rate (Fig. 1). We have compared the increase in fruit length between anthesis and 3, 5 and 7 DPA (Fig. 2). Results indicate that the most parthenocarpic cultivars grew faster and that the greatest differences between cultivars occur at 5 and 7 DPA (Fig. 2). Between anthesis and 3 DPA many cultivars showed an increase in fruit length, but in some growth was arrested after 3DPA, indicating that the increase in length at 3DPA is not a suitable parameter to distinguish between parthenocarpic and non-parthenocarpic cultivars (Fig. 2). However, if parthenocarpic cultivars are those whose fruits reach marketable size at 7 DPA, the increase in fruit length between anthesis and 5 DPA or between anthesis and 7 DPA proves to be a simple and useful parameter for the identification of parthenocarpy in a given genotype (Fig. 2).

Correlation between parthenocarpy and ethylene production and sensitivity

In a previous work the parthenocarpy of the *Cavili* cultivar was found to be associated with a downregulation of ethylene production and signaling in the unpollinated ovary/fruit during the days immediately after anthesis, which was concomitant with a conversion of female into bisexual flowers and the conversion of monoecious into partially andromonoecious plants (Martinez et al, 2013). To extend this study we have selected six parthenocarpic and six non-parthenocarpic genotypes from the studied *C. pepo* collection, and determined the possible correlation of parthenocarpy with either the sexual phenotype of the plants or with ethylene production and sensitivity in different organs.

The andromonoecious index (AI) in each cultivar, based on the degree of stamen development in 5 pistillate flowers of at least 10 plants of each cultivar (see Materials and Methods section) was not correlated with parthenocarpy in all the analyzed cultivars (Fig. 3). In the hybrids *Cavili*, *Parthenon* and *Argo*, but also in landrace *V-185*, the parthenocarpic development of the fruit was associated with the development of stamen in pistillate flowers and with AI higher than 1, but this association was not found in the parthenocarpic landraces *PI261610* and *CpCAL112* (Fig. 3). The latter cultivars

therefore represent one or two new sources of parthenocarpy to add to current commercial hybrids of Zucchini.

The profile of ethylene production in pollinated and unpollinated fruits of each cultivar differed between parthenocarpic and non-parthenocarpic materials. In the non-parthenocarpic cultivars such as *CpCAL044*, pollination maintained or even reduced the production of ethylene during the days immediately after anthesis, while the lack of pollination induced a boost in ethylene production at 3 DPA (Fig. 4). However, in the parthenocarpic cultivars this did not occur, and ethylene production remained at a low level in both pollinated and unpollinated fruits (Fig. 4). These data indicate that fruit set and early fruit development are correlated with a low level of ethylene at 3 DPA, while the induction of ethylene production at 3 DPA appears to be a signal that precedes abortion and senescence of unpollinated fruits in non-parthenocarpic cultivars.

We have found a high negative correlation between parthenocarpy, expressed as the fruit length growth rate of unpollinated fruits during the first 7 DPA, and ethylene production at 3 DPA (Fig. 5). The fruit of parthenocarpic cultivars produced less than 60 nL ethylene/g FW, whereas the fruit of the non-parthenocarpic cultivars produced up to 180-190 nL/g FW (Fig. 5). Given that this peak of ethylene production at 3 DPA is practically absent in the unpollinated fruits of the parthenocarpic cultivars, the lower production of ethylene in the parthenocarpic fruits during the days immediately after anthesis could be a good marker for the identification and selection of parthenocarpic genotypes in *C. pepo*.

Given that the production of ethylene at 3 DPA is a late parameter to use as a marker for selection, we have analyzed whether the production of ethylene in leaves or the ethylene sensitivity of male flowers during earlier stages of plant development were also correlated with fruit set and development. Figure 6 shows the data of ethylene production in young leaves from plants which had already developed 10 true leaves. Although some parthenocarpic cultivars such as *Cavili*, *CpCAL112* and *V-185* showed reduced ethylene production in leaves, others like *Argo* and *Parthenon* produced a high level of ethylene (Fig. 6), indicating that the reduced level of ethylene in the unpollinated fruits of parthenocarpic cultivars is not maintained in other plant organs,

but rather is specific to this developmental process. The production of ethylene in the leaves of the non-parthenocarpic cultivars was very diverse, ranging from 27 to 151 nL/g FW. Therefore, the production of ethylene in leaves cannot be used as a marker for the selection of parthenocarpy in squash.

The sensitivity to ethylene in each cultivar was assessed by determining the effect of external ethylene treatments on the abscission time of male flowers. Male flowers at the same developmental stage of each cultivar were treated with air (control) or ethylene for 48 h, and the number of flowers that reached abscission was recorded every 12 h for 3 days following the treatment. The sensitivity to ethylene was expressed by comparing the times at which abscission occurred in ethylene treated flowers and in controls of the same cultivar. The regression analysis (R= -0.690; p=0.0113) demonstrated a significant negative correlation between the level of parthenocarpy in each cultivar and sensitivity to ethylene (Fig. 7), but correlation was not as high as in the production of ethylene in fruits at 3DPA. In fact, the parthenocarpic landraces *CpCAL112* and *V-185*, as well as the parthenocarpic hybrids *Cavili*, *Parthenon* and *Argo*, showed a reduced response to ethylene, with values of below 20%, while the parthenocarpic cultivar *PI261610* was found to be highly sensitive to ethylene (Fig. 7). Moreover, all non-parthenocarpic cultivars, except *AFR-12*, showed ethylene sensitivity values of over 25%. Consequently, although parthenocarpy in squash appears to be correlated with reduced ethylene sensitivity, this rule does not hold true for certain cultivars.

Discussion

Sources of parthenocarpy for Zucchini breeding programs

With a view to searching for new sources of parthenocarpy that are useful for current Zucchini breeding programs in off-season greenhouse production, we have analyzed 45 accessions with elongated fruits from the core collection of the COMAV germplasm bank at the Polytechnic University of Valencia (Spain) and from the BSUAL at the University of Almería (Spain). In concordance with other previous studies (Om and Hong, 1988; Robinson and Reiners, 1999) under greenhouse environmental conditions, the highest parthenocarpic potential was detected in cultivars of the morphotype Zucchini. In fact, four landraces that showed parthenocarpic potential in the trials of

both 2009-10 and 2011, *CpCAL112*, *PI261610*, *V-185* and *E-27*, all belonged to the morphotype Zucchini, and only one of the detected parthenocarpic cultivars, *CM-37*, belonged to the morphotype Vegetable Marrow (Table 1 and Figs. 1 and 2). Although a certain level of parthenocarpy had been reported in some cultivars of the Cocozelle group (Robinson and Reiners, 1999), but we found no evidence to this (Table 1). Moreover, all the parthenocarpic cultivars detected in this paper produced dark green fruits, with the exception of *Cavili*, which is a commercial white-fruited hybrid. Nijs and Zanten (1982) and Robinson and Reiners (1999) also noted that Zucchini types with dark green fruit had the highest level of parthenocarpy, although not all dark green Zucchini cultivars had good parthenocarpic fruit set either in this work or in previous studies.

The parthenocarpic cultivars detected in this work are not all of the same origin. Four of the identified parthenocarpic cultivars, *CM-37*, *PI261610*, *E-27* and *V-185*, as well as six cultivars that have been classified as non-parthenocarpic, had been previously included in genetic diversity studies of Spanish landraces of squash (Ferriol et al, 2003; Formisano et al, 2012). The parthenocarpic accessions *E-27*, *CM-37*, *PI261610*, *V-185* of our study all belong to subspecies *pepo*, and were grouped close to each other when analyzed by SRAP molecular markers (Ferriol et al, 2003). These Spanish accessions are open pollinated and have been traditionally developed by farmers, which would also explain the diversity of morphotypes detected in some of the accessions (Ferriol et al, 2003). Based on the study of Formisano et al (2012) it was possible to separate two of the identified parthenocarpic accessions, *E-27* and *V-185*. While *E-27* was grouped among the traditional cultivars alongside others such as the non-parthenocarpic *AFR-12*, *V-185* was clustered in the branch of the commercial cultivars (Formisano et al, 2012). These data indicate that at least the parthenocarpy of these two accessions has a different origin, providing evidence, therefore, of two different sources of parthenocarpy and genetic variability for current Zucchini breeding programs. The phenotypic analysis carried out in this work also showed the existence of different sources of parthenocarpy among selected squash cultivars. Thus, parthenocarpic cultivars can be easily separated according to their monoecy stability or andromonoecy index (Fig. 3). The parthenocarpic fruit growth potential of the hybrid *Cavili* is known to be associated with a partial conversion of female into bisexual flowers and therefore

with a conversion of monoecious into partially andromonoecius plants (Martínez et al, 2013). The results presented in Fig. 3 demonstrated that the parthenocarpy of the other two parthenocarpic hybrids, *Argo* and *Parthenon*, as well as that of landrace *V-185*, is related to a high andromonoecious index, i.e. a high conversion of female into bisexual flowers. Therefore, the parthenocarpy of the commercial hybrids and *V-185* appears to have the same origin and is associated with andromonoecy. In these cultivars, parthenocarpic fruit is developed from a bisexual flower, and their floral organs remain attached to the fruit even after harvesting. These fruits with attached flowers occur because of a delay in floral organ maturation and abscission, caused by a reduction in flower ethylene production (Peñaranda et al, 2007; Martínez et al, 20013). The attached flower is, however, an undesirable trait for Zucchini fruit exportation, since the senescence of floral organs, or the wound left after their manual removal, produce a rapid decay of the fruit during postharvest storage (Payán et al, 2006). Nevertheless, we have also identified cultivars, such as *CpCAL112* and *PI261610*, which are completely stable for monoecy and showed no conversion of female into bisexual flowers, and consequently produced no fruit with attached flowers (Fig. 3). These results indicate that not all sources of parthenocarpy in *C. pepo* are dependent on andromonoecy, and that some of the identified accessions constitute new sources of variability for this trait in Zucchini squash.

Relationship between parthenocarpy and ethylene production and sensitivity

The relationship between ethylene and fruit set has been studied in *Arabidopsis* (Carbonell-Bejarano et al, 2011) and *Pisum sativum* (Orzaez and Granell, 1997) by studying the effects of ethylene inhibitors on fruit growth. Treatments extended the time of response to GAs and delayed ovarian senescence in both species, resulting in an increase in the final size of the fruit. The ethylene insensitive mutants of *Arabidopsis* also have a larger window of response to GA than wild type plants (Carbonell-Bejarano et al, 2011). Furthermore, massive expression analyses in tomato have shown a down-regulation of ethylene biosynthesis and signaling genes after fruit set via pollination/fertilization or GA treatments (Vriezen et al, 2008; Pascual et al, 2009). Pascual et al (2009) also observed a differential expression pattern of ethylene genes in *pat3*/*pat4* mutants of tomato, suggesting that ethylene regulates carpel development and parthenocarpic fruit set. In *C. pepo*, we have recently demonstrated that ethylene is

directly involved in fruit set, and that the initiation of fruit growth in this species requires low levels of ethylene production and signaling in the fruit during the days immediately after anthesis (Martínez et al, 2013). This reduced ethylene production is associated with partial andromonoecy and parthenocarpy in the unpollinated fruits of the hybrid *Cavili*, but can also be maintained via pollination or auxin treatments in non-parthenocarpic cultivars (Martínez et al, 2013).

In this paper we demonstrate that in the unpollinated fruits of parthenocarpic cultivars other than *Cavili*, including the commercial hybrids *Argo* and *Parthenon*, and the landraces *CpCAL112*, *V-185* and *PI261610*, ethylene is not induced in the days after anthesis. By contrast, in the unpollinated fruits of the non-parthenocarpic cultivars, including *CpCAL110*, *PI169462*, *CpCA097*, *AFR-12*, *CpCAL003* and *CpCAL044*, the abortion of fruit growth is coupled with a peak of ethylene production at 3 DPA. Consequently, the regression analysis comparing ethylene production in fruits at 3 DPA and the fruit growth rate in 6 non-parthenocarpic and 6 parthenocarpic cultivars of *C. pepo* has demonstrated the existence of a negative correlation between the two characteristics (R=-0.799, p-value=0.0018) (Fig. 5). In this sense the parthenocarpic hybrid *Cavili* is not exceptional, because it is likely that the parthenocarpic development of the fruit in this species is always accompanied by a reduction of ethylene during the days immediately after anthesis, while the increase of ethylene would be a signal that triggers fruit abortion and senescence in absence of natural parthenocarpy or pollination. Therefore, the measurement of ethylene production in the unpollinated fruits of Zucchini at 3 DPA could be a suitable marker for the identification and selection of parthenocarpic materials in this species.

The level of endogenous ethylene has been also found to be associated with sex phenotypes in cucurbit species. Gynoecious lines of melon and cucumber produce more ethylene than monoecious ones, and the level of ethylene production in monoecious cucumber lines increases upon transition from the male to the female phase of development in both apical shoots (Byers et al, 1972b; Rudich, 1990; Owens et al, 1980; Yamasaki et al, 2001; Saito et al, 2007) and in leaves (Martínez et al, 2008). Moreover, ethylene production and sensitivity in seedlings was found to be correlated

with sex expression in *C. pepo* (Manzano et al, 2011). These data suggest that the production of ethylene in vegetative organs could be used as a marker for sex expression in cucurbits. We have analyzed the possible correlation between parthenocarpy and early measurements of ethylene production and sensitivity in vegetative organs and in male flowers. We found that ethylene production in young leaves at early stages of plant development was not correlated with parthenocarpic fruit growth rate in the different cultivars. Ethylene sensitivity, measured as the ethylene response to male flower abscission, was significantly correlated with parthenocarpy, with most parthenocarpic cultivars being less sensitive to ethylene. Nevertheless, one parthenocarpic cultivar (*PI261610*) showed the same sensitivity as non-parthenocarpic ones (Fig. 7), and one non-parthenocarpic cultivar (*AFR-12*) showed the same ethylene sensitivity as parthenocarpic ones. On the basis of these results we concluded that neither ethylene production in leaves nor ethylene sensitivity in male flowers are unambiguous parameters to determine the parthenocarpic potential of a Zucchini cultivar.

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Figure legend

Figure 1. Evolution of parthenocarpic fruit length in 20 cultivars of *C. pepo* during the first 7 days post-anthesis (DPA). The fruit length at each control point represents the mean of at least 10 unpollinated fruits from the same cultivar. The cultivar-specific fruit growth rates allowed the separation of parthenocarpic and non-parthenocarpic cultivars in squash. In the non-parthenocarpic cultivars fruit growth aborted after 3 DPA, while in the parthenocarpic cultivars fruits maintained their growth and reached marketable size before or at 7 DPA.

Figure 2. Increase in the mean length of parthenocarpic fruits from anthesis to 3, 5 and 7 DPA in 20 cultivars of *C. pepo* ssp. *pepo*. Data indicate the mean of at least 10 fruits per cultivar.

Figure 3. Regression of Andromonoecy Index (AI) onto pathenocarpic fruit growth rate during the first 7 DPA in six parthenocarpic and six non-parthenocarpic cultivars of *C. pepo*. Although the parthenocarpy of the hybrids *Argo, Cavili* and *Parthenon* is correlated with AI higher than 1, i.e. a partial conversion of female into bisexual flowers, the parthenocarpic potential of cultivars *CpCAL112* and *PI261610* is not associated with andromonoecy (AI=0). Horizontal and vertical bars represent standard errors for AI or parthenocarpic fruit growth rate, respectively.

Figure 4. Evolution of ethylene production in pollinated and unpollinated ovaries/fruits of two cultivars of *C. pepo* that differ in their parthenocarpic potential. (A) *CpCAL044*, which has been classified as non-parthenocarpic, and (B) *V-185*, which has been classified as parthenocarpic. Bars represent standard errors (n=3). Ethylene production profiles in other parthenocarpic and non-parthenocarpic cultivars were similar.

Figure 5. Regression of ethylene production in unpollinated fruits at 3 DPA onto fruit length growth rate for the first 7 DPA in six parthenocarpic and six non-parthenocarpic cultivars of *C. pepo*. The linear regression analysis (R= -0.799, p=0.0018) indicates a strong correlation between variables with a significance level of 99%. Horizontal and vertical bars represent standard error for ethylene production and fruit growth rate, respectively, in each cultivar.

Figure 6. Regression of ethylene production in young leaves onto fruit length growth rate during the first 7 DPA in six parthenocarpic and six non-parthenocarpic cultivars of *C. pepo*. The lineal regression analysis (R= -0.420; p=0.1700) denotes no correlation between variables with a level of significance of 90%. Horizontal and vertical bars represent standard error for ethylene production and fruit growth rate, respectively, in each cultivar.

Figure 7. Regression of ethylene sensitivity (expressed as the percentage of time at which the abscission of ethylene treated male flowers is reduced respect to control non-treated flowers) onto fruit length growth rate during the first 7 DPA in six parthenocarpic and six non-parthenocarpic cultivars of *C. pepo*. The lineal regression analysis (R= -0.699, p=0.0113) indicates a significant correlation between the two variables, with parthenocarpic cultivars showing reduced ethylene sensitivity. Horizontal and vertical bars represent standard error for ethylene sensitivity and fruit growth rate, respectively, in each cultivar.

Table 1. Cultivars of *C. pepo* morphotypes Zucchini, Vegetable Marrow and Cocozelle.

Cultivar Name	Morphtype[1]	Parthenocarpy score (Mean±S.D.)[2]	Sex phenotype[3]	Growth habit[4]	Source[5]	Selection in the first screening	Country of origin[5]	Region
ARGO	Z	4.50±1.00	PA	B		Yes		
CAVILI	Z	4.75±0.70	PA	B		Yes		
PARTHENON	Z	4.40±1.20	PA	B		Yes		
435	Z	1.50±1.00	M	B	COMAV		Spain	Castilla La Mancha
942	Z	2.25±0.35	M	B	COMAV		Spain	Castilla La Mancha
BLACK BEAUTY	Z	2.25±0.50	M	B	COMAV			
C-3	Z	2.00±1.00	M	B	COMAV		Spain	Cataluña
C-68-1	Z	2.33±0.58	M	B	COMAV		Spain	Cataluña
CA-82	Z	2.40±1.94	PA	B	COMAV		Spain	Canarias
CL-21	Z	2.00±0.00	M	V	COMAV		Spain	
CM-39	Z	2.00±0.00	M	V	COMAV		Spain	Castilla La Mancha
CM-47	Z	2.00±1.41	M	B	COMAV		Spain	Castilla La Mancha
CpCAL003	Z	1.33±0.58	M	B	BSUAL	Yes	Spain	Andalucía
CpCAL110	Z	4.00±1.00	M	B	BSUAL	Yes	Spain	Andalucía
CpCAL112	Z	4.50±0.71	M	B	BSUAL	Yes	Spain	Andalucía
CpCAL044	Z	3.50±1.29	PA	B-V	BSUAL	Yes	Spain	Valencia
E-27	Z	3.33±0.58	M	B	COMAV	Yes	Spain	Extremadura
GRECIA6	Z	3.00±0.82	M	B	COMAV	Yes	Greek	Agias Paraskeis
MUC-16	Z	1.67±0.58	M	B	COMAV		Spain	Murcia
PASCUAL40	Z	3.33±0.58	PA	B	COMAV	Yes	Spain	Valencia
PASCUAL77	Z	2.33±0.58	PA	B	COMAV		Spain	Valencia
PI261610	Z	3.00±0.00	M	B	USDA	Yes	Spain	Castilla Leon
S4	Z	1.00±1.00	PA	V	COMAV	Yes	Spain	Cantabria
V-4	Z	2.33±0.58	M	B	COMAV		Spain	Valencia
V-50	Z	2.00±0.00	M	B	COMAV		Spain	Valencia
PI379307	Z	2.00±0.00	M	B	USDA		Yugoslavia	
CL-20	Z	1.75±0.50	M	V	COMAV		Spain	Castilla Leon
V-116	Z-CO	2.67±1.15	PA	B	COMAV		Spain	Valencia
V-185	Z-CO	3.33±1.15	PA	B-V	COMAV	Yes	Spain	Valencia
AFR-12	Z-VM	3.00±1.00	PA	B	COMAV	Yes	Morocco	Khmelat
A-17	Z-VM	2.00±0.00	M	V	COMAV		Spain	Aragón
C-1	Z-VM	2.00±1.41	PA	B	COMAV		Spain	Cataluña
C-9	Z-VM	2.33±0.58	PA	V	COMAV		Spain	Cataluña
V-171	Z-VM	1.25±0.96	PA	V	COMAV	Yes	Spain	Valencia
V-32	Z-VM	2.33±0.58	M	V	COMAV		Spain	Valencia
CpCAL097	CO-VM	1.00±1.00	PA	V	BSUAL	Yes	Spain	Valencia
V-117	CO	2.67±0.57	PA	V	COMAV		Spain	Valencia
CA-154	VM-CO	1.33±1.04	M	V	COMAV	Yes	Spain	Canarias

Cultivar Name	Fruit morphtype[1]	Parthenocarpy value (Mean±S.D.)[2]	AI (Mean±S.D.)[3]	Growth habit[4]	Source[5]	Selection	Country of origin[5]	Region
CM-37	VM	2.75±0.50	M	B	COMAV	Yes	Spain	Castilla La Mancha
CpCAL005	VM	1.25±0.50	PA	V	BSUAL	Yes	Spain	Andalucía
CA-84	VM	2.00±1.00	PA	B	COMAV		Spain	Canarias
A-19	VM	2.00±0.00	PA	V	COMAV		Spain	Aragón
AN-116	VM	2.00±0.00	PA	V	COMAV		Spain	Andalucía
AN-92	VM	2.33±0.57	PA	B	COMAV		Spain	Andalucía
C-72B	VM	1.67±1.52	M	B	COMAV		Spain	Cataluña
CA-35	VM	1.67±0.58	M	B	COMAV		Spain	Canarias
PI169462	VM	3.00±0.82	PA	B	USDA	Yes	Turkey	Canakkale
S-2	VM	2.25±1.77	M	V	COMAV		Spain	Cantabria

[1]Fruit shape morphotypes: Z: Zucchini, VM: Vegetable Marrow, CO: Coccozelle

[2]Parthenocarpy scores represent the mean ± standard deviation of at least 3 evaluations. Values are the mean scores from 0 to 5 of parthenocarpic potential of each cultivar (see Material and Methods). Selected parthenocarpic cultivars were those with a mean scores above 2.75. Non parthenocarpic cultivars with mean scores below 1.50 were also selected as negative control.

[3]Sex phenotype.PA: Partially andromonoecious, M: Monoecious.

[4]Growth habit: B: bushy, V: vine

[5]COMAV: Germoplam Bank at the Polytechnic University of Valencia; USDA: United States Department of Agriculture; BSUAL: Seed Bank at the University of Almería.

[6]Origin data obtained from the COMAV (http://www.comav.upv.es/BancoGermoplasmaUPV/consulta2sesion.php) and USDA/GRIN (http://www.ars-grin.gov/cgi-bin/npgs/html/tax_acc.pl) germplasm banks and BSUAL.

700

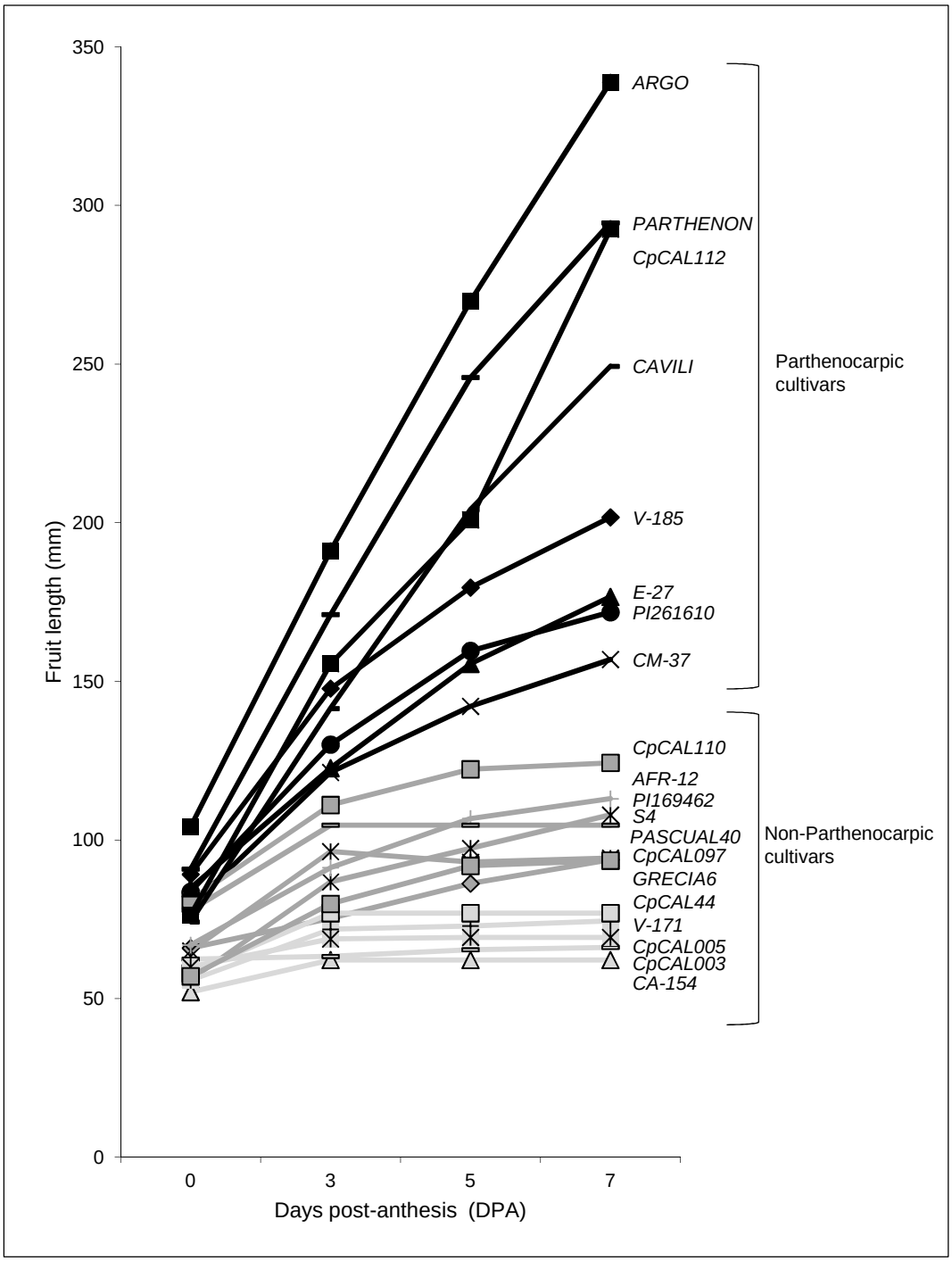
350
300
250
200
150
100
50
0
Fruit length (mm)
0
3
5
7
Days post-anthesis (DPA)
ARGO
PARTHENON
CpCAL112
CAVILI
V-185
E-27
PI261610
CM-37
CpCAL110
AFR-12
PI169462
S4
PASCUAL40
CpCAL097
GRECIA6
CpCAL44
V-171
CpCAL005
CpCAL003
CA-154
Parthenocarpic cultivars
Non-Parthenocarpic cultivars

Fig. 1

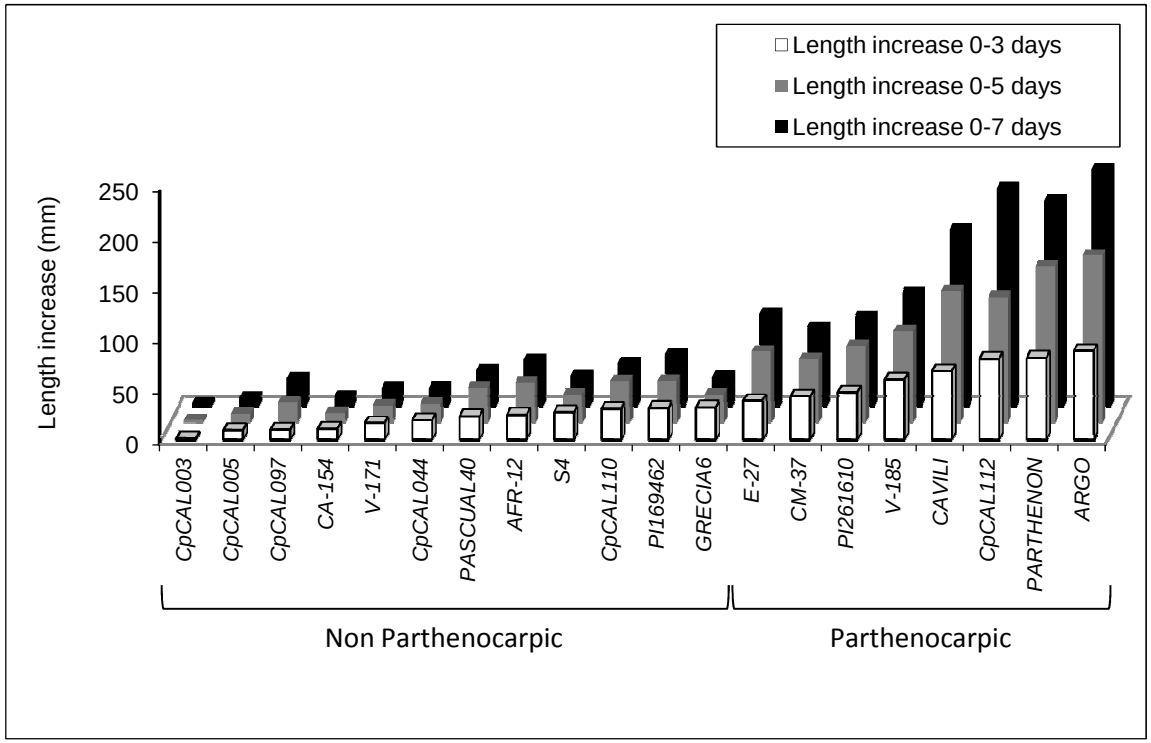
Length increase 0-3 days
Length increase 0-5 days
Length increase 0-7 days
Length increase (mm)
250
200
150
100
50
0
CpCAL003
CpCAL005
CpCAL097
CA-154
V-171
CpCAL044
PASCUAL40
AFR-12
S4
CpCAL110
PI169462
GRECIA6
E-27
CM-37
PI261610
V-185
CAVILI
CpCAL112
PARTHENON
ARGO
Non Parthenocarpic
Parthenocarpic

Fig. 2

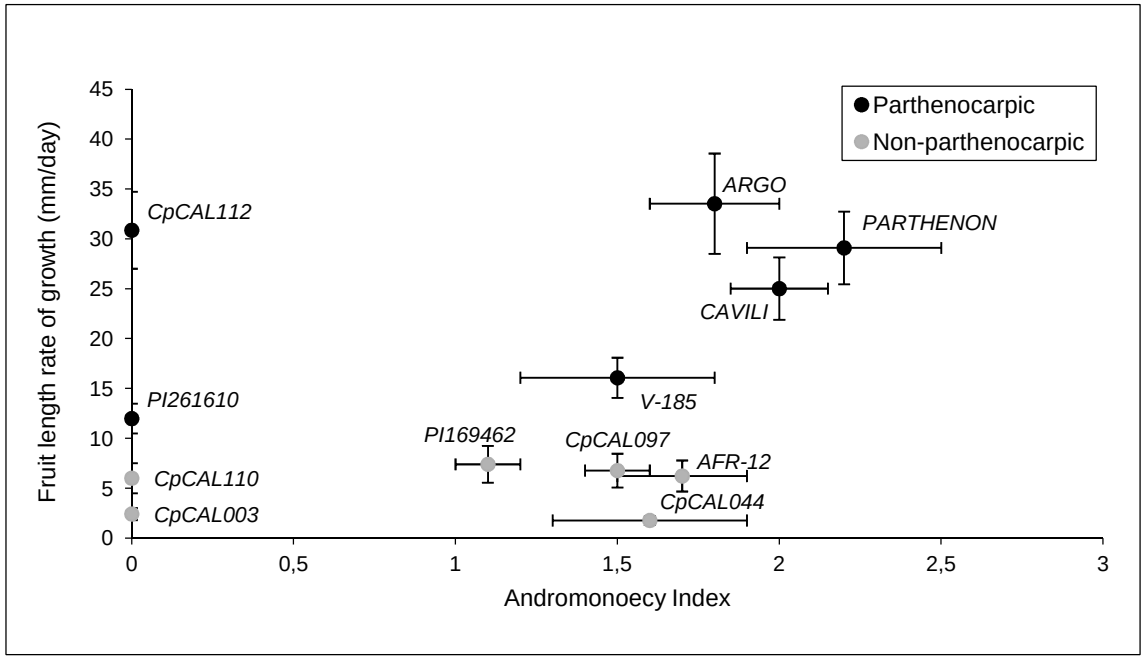
Parthenocarpic
Non-parthenocarpic
Fruit length rate of growth (mm/day)
Andromonoecy Index
45
40
35
30
25
20
15
10
5
0
0
0,5
1
1,5
2
2,5
3
ARGO
PARTHENON
CAVILI
V-185
CpCAL112
PI261610
PI169462
CpCAL097
AFR-12
CpCAL110
CpCAL044
CpCAL003

Fig. 3

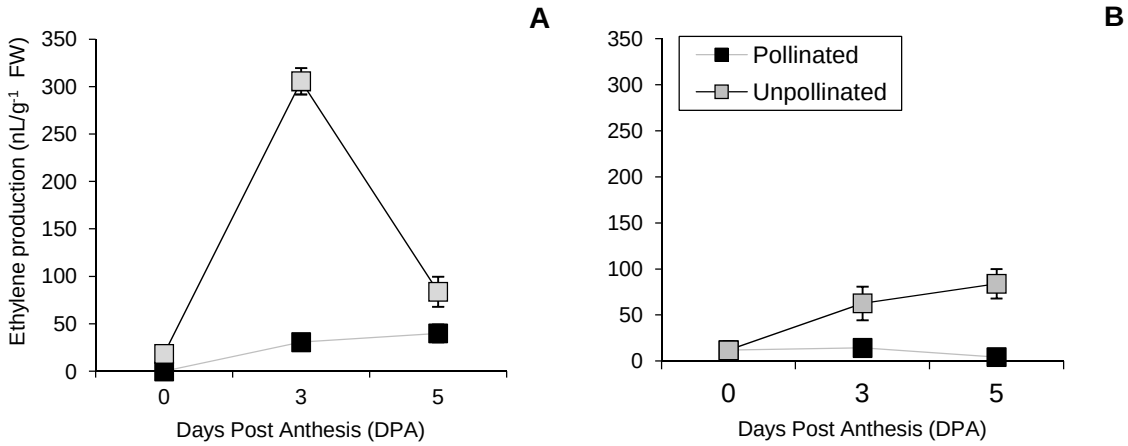
A
B
Ethylene production (nL/g-1 FW)
350
300
250
200
150
100
50
0
0
3
5
Days Post Anthesis (DPA)
Pollinated
Unpollinated

Fig. 4

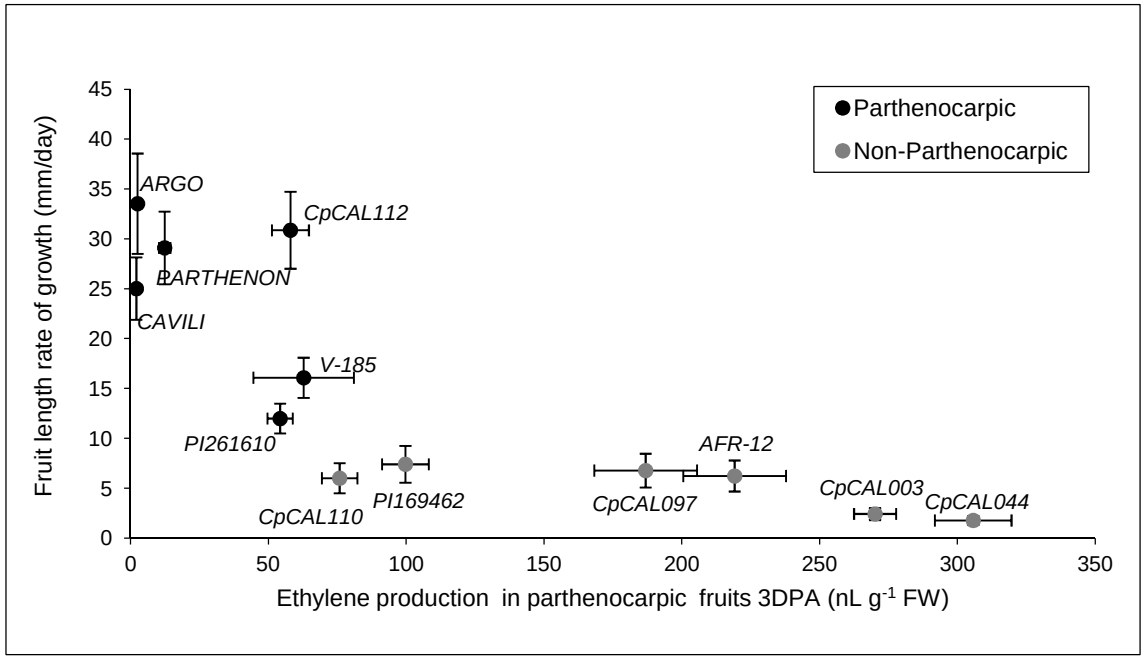
Parthenocarpic
Non-Parthenocarpic
Fruit length rate of growth (mm/day)
Ethylene production in parthenocarpic fruits 3DPA (nL g-1 FW)
ARGO
CpCAL112
PARTHENON
CAVILI
V-185
PI261610
AFR-12
CpCAL003
CpCAL044
CpCAL097
PI169462
CpCAL110
0
5
10
15
20
25
30
35
40
45
0
50
100
150
200
250
300
350

Fig. 5

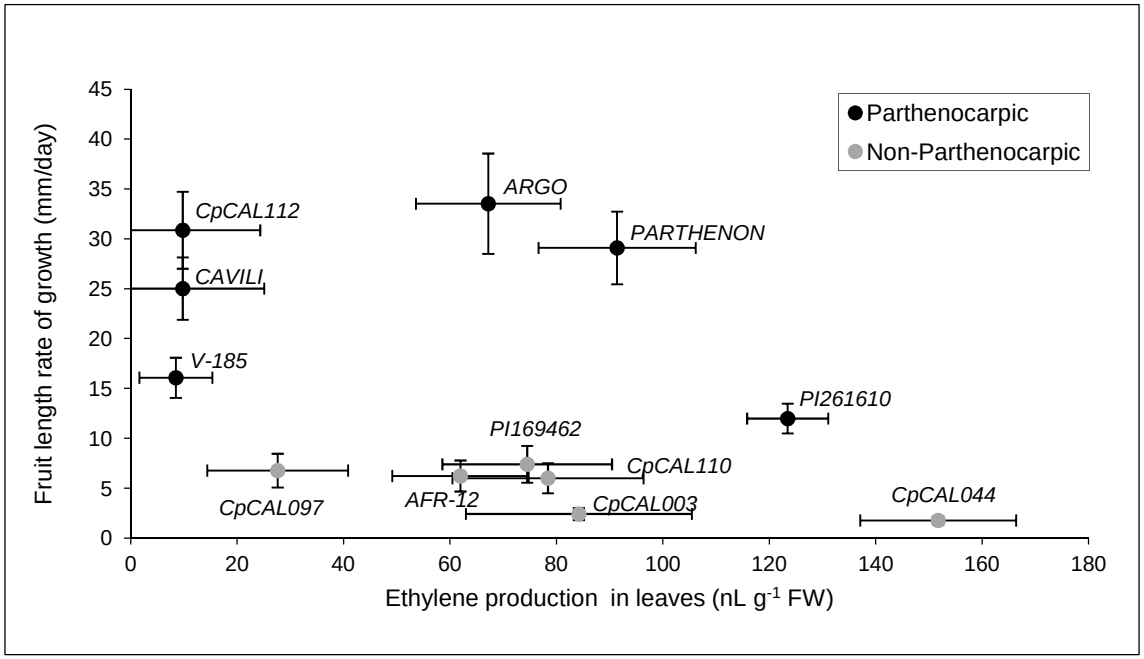
Parthenocarpic
Non-Parthenocarpic
Fruit length rate of growth (mm/day)
Ethylene production in leaves (nL g-1 FW)
CpCAL112
CAVILI
V-185
CpCAL097
ARGO
PARTHENON
PI169462
CpCAL110
AFR-12
CpCAL003
PI261610
CpCAL044
0
5
10
15
20
25
30
35
40
45
0
20
40
60
80
100
120
140
160
180

Fig. 6

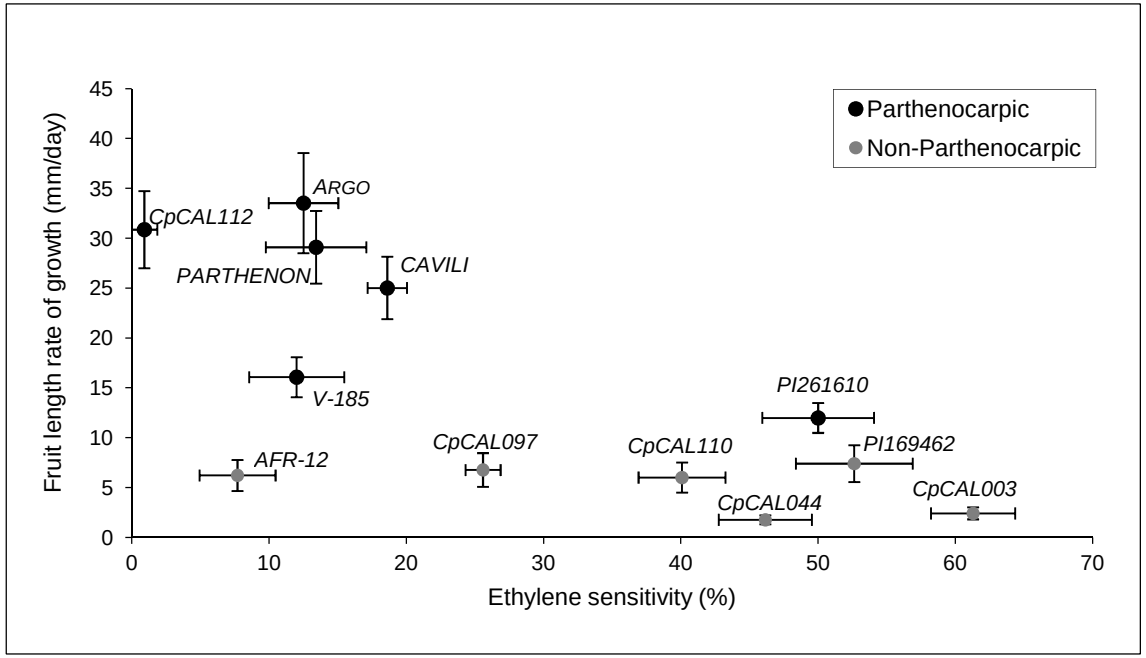
Parthenocarpic
Non-Parthenocarpic
Fruit length rate of growth (mm/day)
Ethylene sensitivity (%)
0
5
10
15
20
25
30
35
40
45
0
10
20
30
40
50
60
70
ARGO
CpCAL112
PARTHENON
CAVILI
V-185
AFR-12
CpCAL097
CpCAL110
PI261610
PI169462
CpCAL044
CpCAL003

Fig. 7