Changes produced by the application of biostimulants on almond rootstocks properties during the nursery process

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Abstract. During the last ten years we have assisted to the consolidation of the almond crop that has remarkably increased its cultivation area causing a high demand for both plants and products related to growth stimulation. Accordingly, in the present work we aim to study the changes produced by the contribution of two biostimulants (humic and fulvic acids or aminoacids) on the properties of almond tree rootstocks. This kind of studies are of interest to the nursery cultivation industry where rapid growth of trees and good adaptation to their cultivation environment are required. Plants' radicular and vegetative systems responded differently according to the rootstock selection. The fastest and vigorous vegetative development was observed in GN rootstock whereas GF 677 showed the greatest number of main roots and RP-R of secondary roots. Differences on antioxidant activity and phenol content have also been found between rootstocks. All the tested samples were found to have a high antioxidant power and a high phenol content but GN stood out in this regard over the other rootstocks under study. The efficiency of the biostimulants applied has been verified. Both biostimulants promoted the development of the aerial part of the trees but biostimulant 2 did it to a greater extent. Biostimulant 1 was able to duplicate the number of main roots in RP-R and during the first year of study, biostimulant 2 originated an increase of the weight of the root system by 26.44% for RP-R, 16.93% for GF 677 and 48.00% for GN. In view of these results, synthetic chemical fertilizers can be at least partially replaced by biostimulants.
Key Words: *Prunus dulcis* Miller, natural fertilizers, vegetative system, radicular system, antioxidant activity, phenolic content.

Introduction. Nuts are world-renowned and valued for their sensory, nutritional and health attributes. On a global basis, almond tree (*Prunus dulcis* Miller) production levels outperform other nuts like pistachio or hazelnuts and are distributed mainly in the regions of California and the Mediterranean Basin. During the last ten years we have witnessed a significant consolidation of this crop, while the cultivation surface has increased by 12% globally, the production of almonds has been increased by 55% (FAOSTAT 2018), placing the almond tree as a very important crop due to the high commercial value of its fruits. The three main objectives that are pursued in the agronomic research for the enhancement of the cultivation of the almond tree are the increase of its performance with self-compatible and late flowering varieties, the improvement in the composition and quality of its fruits and an improved answer to biotic and abiotic stresses (Saa et al 2015).

The use of rootstocks is an important tool used by nurseryman in the improvement of crop adaptation to certain stresses, especially in situations of stakeout (Mondragón-Valero et al 2017). Rootstocks can modify the eco adaptability of the cultivars so that, an accurate characterization of them is essential to identify the best cultivar-rootstock combinations for each environment with the objective of obtaining high quality crops.

Often, the rootstock characterization is limited exclusively to their morphometric properties and how they influence the grafted varieties. However, the usual morphometric characterization should be accompanied by a chemical analysis that allows

knowing how the chemical composition influences the antioxidant capacity and its enzymatic and non-enzymatic system (Zrig et al 2011). These chemical properties are important as they can influence the defense mechanisms of the plant by inducing resistance to certain stresses, pathogens or diseases. The phenolic compounds are a good example of this, they are situated in the different tissues of the plants and ensure some protection against biotic and abiotic stresses (Tenhaken 2015). The contribution of phenols in resistance to plant diseases is largely based on their cytotoxicity, they are known antioxidants and have been shown to elicit cellular responses that are triggered to counteract oxidative stress (Agati et al 2011) so a more accurate information about the phenolic content of the different rootstocks and their antioxidant activity can be helpful in almond improvement programs.

Another of the most used tools in the nursery industry in order to offer quality plants is the use of fertilizers. There is however a lack of nurseries in the world that produce fruit trees using sustainable management techniques (Grzyb et al 2015). It is known that proper nutrient management is crucial to optimize the production of fruit crops, however, fruit producers usually apply to the substrate large amounts of chemical fertilizers, sometimes higher than those that are really needed, causing high environmental pollution impact (Tanou et al 2017). Some years ago, certain nurseries undertook the attempt to use sustainable products such as biostimulants based on mycorrhizal funghi, humus, seaweed extract and humic and fulvic acids to replace or complement the most polluting traditional products. It was concluded that some of these products allow an adequate development of the seedlings, guaranteeing a correct establishment in the future orchard (Grzyb et al 2015). The use of biostimulant products is considered as an innovative alternative to address the challenges of sustainable agriculture due to its ability to improve nutrient absorption, stimulate the development of the plant, minimize the use of fertilizers (Povero et al 2016; Yakhin et al 2017) and to induce tolerance to stresses produced by the environment especially drought and salinity (Posmyk & Szafrańska 2016). It should be noted however that biostimulant origins are very diverse and the mode of action of many of them is still under study (Povero et al 2016; Colla et al 2017) and that the results obtained by biostimulants are often dependent on the type of crop and the environment (Yakhin et al 2017) so it is key to continue investigating its use.

The objective pursued in this study is to characterize both morphometrically and chemically three almond rootstocks to have a greater knowledge of their possibilities of eco-environmental adaptation. In addition, we intend to study its response at both vegetative and radicular levels against the contribution of different root biostimulants to increase the eco adaptability of the plant in its nursery phase.

Material and Method

Vegetal material and growth conditions. The trials were carried out on the east zone of Spain, more specifically in the province of Valencia, at the facilities of the Universitat Politècnica de Valencia (latitude 39º28'50''N, longitude 0º21'59''W). The average annual temperatures of the region are 18.3ºC with maximum average temperatures in the month of August (30.2ºC) and minimum temperatures in the month of January (7.1ºC). The average annual rainfall is 475 mm with a relative humidity of 65% (AEMET 2019).

To carry out the trial, a total of 90 almond trees were characterized under UPOV norm and obtained from a certified nursery in phenological stage 10 of the BBCH scale. Thirty individuals corresponded to the rootstock GF 677 (*Prunus persica* L. × *Prunus dulcis* M.), 30 individuals with the G×N Garnem® (GN) rootstock (*Prunus dulcis* M. (cv Garrigues) × *Prunus pérsica* L. (cv. Nemared)) and another 30 with the ROOTPAC® (RP-R) rootstock (*Prunus cerasifera* E. × *Prunus dulcis* M.).

The rootstocks were transplanted into pots of 80 L capacity with a substrate prepared on demand composed of 25% silica, 38% vaporized peat and 37% sand. The irrigation dose was 40 L of water per month distributed in irrigations of 40 min on alternate days with a self-compensating and anti-draining dripper of 4 L h^{-1} (uniformity coefficient of 85%).

Regarding fertilization, each of the three rootstocks was subjected to three types of treatment:

- 10 specimens of each rootstock were treated with biostimulant 1;
- 10 specimens of each rootstock were treated with biostimulant 2;
- 10 specimens of each rootstock were taken as control trees, they did not have any contribution of fertilizer and only water was provided.

The chemical composition of the biostimulants in showed on Table 1. The treatments were applied around the area of influence of the roots on a weekly basis at a rate of 8 cm^3 of biostimulant per tree and week during the two years of study.

Table 1

Chemical compositions of the biostimulants applied

Biostimulant 1		*Biostimulant 2*	
"L" free aminoacids	4.7% w/w	Total humic extract	25% w/w
Nitrogen (N)	5.5% w/w	Fulvic acids	25% w/w
Potassium (K_2O)	1% w/w	Nitrogen (N)	4% w/w
Organic matter	22% w/w	Phosphorus (P_2O_5)	0.5% w/w
Fe-HEDTA	0.5% w/w	Potassium (K_2O)	0.5% w/w
Weed extract	4% w/w	Organic matter	45% w/w
Zeaxanthins	0.07% w/w		

The total duration of the trial was two years, from July 2015 to July 2017. At the end of the first-year period (July 2016), a sample of 45 individuals were taken for data collection corresponding to 15 specimens of GF 677 (5 units treated with biostimulant 1, 5 units with biostimulant 2 and 5 control units), 15 specimens of GN (5 units treated with biostimulant 1, 5 units with biostimulant 2 and 5 control units) and 15 specimens of RP-R (5 units treated with biostimulant 1, 5 units with biostimulant 2 and 5 control units). The rest of the individuals were left under the initial conditions for another year. This schedule allows us to compare the development of the trees over a total period of two years.

Morphometrical characterization. Regarding the aerial part of the samples, the influence of the different rootstocks and biostimulants consisted on the evaluation of the height of the tree, the trunk height and the weight of the shoots. The diameter of the graft area was also measured since it is the graft-rootstock union zone and therefore the most sensitive area in the nursery years. The parameters studied in the radicular system were, the number of main roots, their length and diameter, and the distance to the first bifurcation. In the same way, the number of secondary roots, their length, diameter and the distance between the beginning of the secondary root and their first bifurcation to tertiary roots were counted. To measure the dry weights of each group of roots (main, secondary, and tertiary) the root systems were introduced in a muffle (Memmert model) at 38ºC until constant weight.

Chemical characterization

Aqueous extracts preparation. An amount of 200 g of adult leaves (BBCH stage 19) were taken from each of the almond rootstock studied. Each of the leaf samples was lyophilized at -60°C (LyoAlfa 6 Telstar, Barcelona, Spain). After the lyophilization process the leaves were crushed to immediately undergo an aqueous extraction following the procedure of Lima et al (2016) which consists of adding 5 g of lyophilized sample (20 mesh) to 250 mL of distilled water and let boil for 45 min. The extract was then filtered with a Whatman filter No. 4 and the aqueous solution was kept frozen until posterior lyophilization process. Once lyophilized, the obtained extracts were dissolved in distilled water at a concentration of 50 mg mL^{-1}. From this concentration were prepared concentrations ranging from 0.01 to 3 mg mL^{-1}, used to determine the content of phenols

and the antioxidant activity of the almond leaf. All measurements were made in triplicate and each test was repeated three times.

Reagents and products. Gallic acid, caffeic acid, hydrochloric acid, free radical 2.2-diphenyl-1-picrylhydrazyl (DPPH), radical ABTS (2,2'-Azinobis-3-ethylbenzthiazoline-6-sulphonic acid), quercetine, potassium persulfate ($K_2S_2O_8$) and ferric chloride [$FeCl_3$ $6H_2O$] were obtained from Sigma-Aldrich (St. Louis, MO, USA). Phosphate buffer (pH 6.6) was prepared with dihydrogen sodium phosphate (NaH_2PO_4 $2H_2O$) and disodium hydrogen phosphate (Na_2HPO_4 $2H_2O$) both from Sigma-Aldrich. Ethanol, methanol and acetone used in the extractions were HPLC grade. The distilled water used was from a Milli-Q water purification system.

Antioxidant activity

a. Antioxidant capacity through DDPH assay. The ability of the sample to scavenge the DPPH free radical (2,2-diphenyl-1-picrylhydrazyl) was studied by the method described by Lima et al (2016). To prepare the sample, 0.3 mL of the extract concentrations described above were added to 2.7 mL of methanolic DPPH solution (6×10-5 mol L^{-1}). The solution was stirred vigorously and kept under dark conditions for one hour to ensure the stability of the absorbance values subsequently measured at 517 nm in a Jenway 6320D spectrophotometer. The ability to neutralize free radicals was measured as a percentage of DPPH decolorization by the following equation:

$$\text{Scavenging activity (\%)} = [(\text{ADPPH- As}) / \text{ADPPH}] \times 100$$

where: As is the absorbance of the sample and ADPPH is the absorbance of the DPPH solution.

The EC_{50} which is the effective concentration at which a 50% inhibition percentage of free radicals occurs was also calculated.

b. Antioxidant capacity through ABTS assay. This method, based on the ability of a sample to inhibit the radical ABTS (2,2'-Azinobis-3-ethylbenzthiazoline-6-sulfonic acid), was carried out based on the protocol described by Lima et al (2016). There was prepared a 25 mL solution of ABTS (7mM) with 440 µL of potassium persulfate ($K_2S_2O_8$) and kept at room temperature under total darkness for 12-16 h to form the radical. A precise volume of the mentioned solution was diluted with ethanol to obtain an absorbance of 0.70±0.02 at a wavelength of 734 nm. Once the radical was formed, we mixed 2 mL of ABTS solution with 0.1 mL of sample and after 6 minutes the absorbance was read at a wavelength of 724 nm. The ability to neutralize free radicals was measured as a percentage of ABTS discoloration using the same equation used for DPPH. The EC_{50} which is the effective concentration at which a 50% inhibition percentage of free radicals occurs was also calculated.

c. Reducing power. The reducing power of the extracts was obtained by the procedure described by Lima et al (2016). Were mixed 1 mL of sample with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and with 2.5 mL of 1% (w/v) K3[Fe(CN)6] solution. The mixture was incubated in a water bath for 20 min at 50°C, and then cooled at room temperature. Subsequently, 2.5 mL of trichloroacetic acid 10% (w/v) was added and vigorously shaken. Were removed 2.5 mL of supernatant to which were added 0.5 mL of 0.1% (w/v) $FeCl_3$ $6H_2O$ solution. After that the absorbance was read at 700 nm. The concentration of extract that allowed an absorbance of 0.5 (EC_{50}) was calculated through the absorbance graph at 700 nm as a function of the different concentrations.

Different phenolic groups content. For the determination of the different phenolic groups, we mixed 1 mL of sample with 1 mL of 96% ethanol (0.1%, 2% HCl) and then added 8 mL of 2% hydrochloric acid (2% HCl). The absorbance was measured at 280 nm to determine simple phenols using gallic acid as standard; at 320 nm to determine the hydroxycinnamic acid derivatives using caffeic acid as standard; and finally at 360 nm to estimate the flavonoids using quercetin as standard. The results of simple phenols were expressed as milligrams of gallic acid equivalent per gram of extract (mg GAE g^{-1}).

Hydroxycinnamic acid derivatives were expressed as milligrams of caffeic acid equivalent per gram of extract (mg CAE g^{-1}) and flavonoids as milligrams of quercetin per gram of extract (mg QE g^{-1}).

Statistical processing. An analysis of variance (ANOVA) with Type III sums of squares was performed using the GLM (General Linear Model procedure) of the Statgraphic X64 software. The normal distribution of the residuals and the homogeneity of variance, were evaluated by means of the Kolmogorov-Smirnov with Lilliefors correction (if n > 50) or the Shapiro-Wilk's test (if n < 50), and the Levene's tests, respectively. All dependent variables were analyzed using a one-way ANOVA with or without Welch correction, depending on whether the requirement of the homogeneity of variances was fulfilled or not. The main factor studied was the effect of rootstocks (GN, 677, and RP-R) as well of the different biostimulants in vegetative and radicular system parameters of the almond trees studied. If a statistical significant effect was found, means were compared using Tukey´s honestly significant difference multiple comparison test or Dunnett T3 test also depending on whether equal variances could be assumed or not. All statistical tests were performed at a 5% significance level.

Results. The use of rootstocks is a fundamental tool in today's fruit production due to its ability to modify the behavior of the cultivars and adapt them to different environments, so it is paramount to characterize them morphometrically and chemically in the nursery phase.

Physical characteristics of the rootstocks. In our trial, the rootstock with the fastest vegetative development in the first year of life of the plant was GN that showed greater vigor than the RP-R and GF 677 with tree heights of 61 cm, 57.60 cm and 46.80 cm respectively, biomass weight and diameter in the grafted area (Table 2). The only difference that was observed at the level of aerial development between the GF 677 and RP-R rootstocks was in the distance from the trunk to the first bifurcation where it was observed that the RP-R showed higher trunk height than the other two rootstocks under study.

The results obtained in the root system of one-year-old trees were much more heterogeneous than those obtained in the vegetative system. As shown in Table 2, the GF 677 rootstock presented a greater number of main roots than the GN and RP-R rootstocks, being observed an inverse relationship between the number of main roots and their diameter, RP-R had a lower number of main roots than the rest of the rootstocks, but of greater thickness. Considering the main roots, GN stood out statistically on the other two rootstocks with 13.30 g weight of main roots RP-R presented a higher number of secondary roots (50.80) than GN (35.20), which in turn obtained a greater number than GF 677 (29.90). Again, an inverse relationship between the number of roots and the average diameter of each root is observed in the secondary roots, as it was the case of the main roots. This same relation is repeated for the weight of the roots, thus presenting the GF 677, the greater weight of secondary roots and RP-R the smaller. Although the average distance from the main roots to the first bifurcation (or secondary roots) was similar for the three rootstocks, statistical differences were observed in the mean distances of the secondary to the tertiary roots. The secondary roots of GN bifurcate deeper than those of GF 677 or RP-R. When analyzing the root system of the one-year samples, the GN rootstock stands out as the one with the highest total weight of roots and RP-R for showing greater maximum length and therefore a higher level of depth exploration.

The results obtained in the two-years-old samples were similar to those obtained in the one-year-old samples. GN continues to stand out at both vegetative and radical levels. It is the most vigorous rootstock with a higher tree height, but above all, with a higher dry weight of young shoots than the rest of the studied rootstocks (Table 2). The greater vigor of GN also moves to the trunk cross-sectional area and to a greater weight of the radical system.

Table 2

Vegetative and radicular parameters of the different rootstocks during the two year-study (n = 10; mean value ± standard deviation)

Year of study	*Year 1*			*Year 2*		
Rootstock	*RP-R*	*GF 677*	*GN*	*RP-R*	*GF 677*	*GN*
Vegetative system						
Tree height (cm)	57.60±1.14^{b}	46.80±0.84^{a}	61.00±0.70^{c}	135.94±2.69^{b}	108.00±4.24^{a}	147.40±32.66^{b}
Trunk longitude (cm)	31.30±7.95^{b}	22.70±0.47^{a}	24.28±0.33^{a}	46.78±11.85^{a}	37.95±21.28^{a}	32.55±20.29^{a}
Young shoots weight (g)	8.39±1.01^{a}	8.04±0.07^{a}	9.33±0.05^{b}	58.57±8.74^{a}	55.15±9.68^{a}	101.65±5.61^{b}
Radicular system						
Diameter of grafted area (mm)	7.72±0.68^{a}	7.96±0.15^{a}	8.82±0.13^{b}	23.55±2.08^{a}	22.05±1.09^{a}	29.17±1.09^{b}
Number of tap roots	7.00±1.30^{a}	9.00±0.55^{b}	8.00±0.54^{ab}	24.00±4.56^{a}	34.00±4.24^{b}	22.50±3.53^{a}
Tap root diameter (mm)	5.31±0.02^{c}	2.82±0.06^{a}	3.82±0.05^{b}	7.22±0.03^{c}	2.99±1.70^{a}	5.86±1.09^{b}
Distance of tap roots to first bifurcation (mm)	1.71±0.72^{a}	1.32±0.02^{a}	1.24±0.02^{a}	13.07±5.52^{a}	10.88±5.49^{a}	8.67±0.32^{a}
Tap root weight (g)	10.75±0.85^{b}	8.36±0.23^{a}	13.30±0.25^{c}	180.70±14.28^{ab}	97.45±6.01^{a}	249.75±139.37^{b}
Number of lateral roots	50.40±3.04^{c}	29.80±1.30^{a}	35.20±1.92^{b}	450.20±27.23^{b}	304.40±96.87^{a}	287.25±141.42^{a}
Lateral root diameter (mm)	0.63±0.29^{a}	1.31±0.02^{b}	0.82±0.03^{a}	0.65±0.30^{a}	0.95±0.02^{ab}	1.26±0.17^{b}
Distance of tap lateral roots to first bifurcation (mm)	4.34±0.96a	4.48±0.02a	5.84±0.04^{b}	13.99±3.09^{a}	18.05±5.6^{a}	15.21±0.75^{a}
Lateral root weight (g)	5.23±0.78^{a}	7.43±0.20^{c}	6.58±0.08^{b}	58.57±8.74^{a}	55.15±9.68^{ab}	101.65±58.61^{b}
Absorbing root weight (g)	4.03±0.55^{c}	1.24±0.16^{a}	3.02±0.06^{b}	12.38±1.71^{b}	6.32±4.27^{a}	6.75±1.09^{a}
Total roots dry weight (g)	11.23±0.56^{a}	10.45±0.16^{a}	13.91±0.06^{b}	232.36±22.38^{ab}	152.60±3.67^{a}	351.40±197.99^{b}
Roots longitude (mm)	920.00±73.31^{b}	758.01±8.40^{a}	762.00±16.40^{a}	1720.41±130.70^{b}	1142.00±282.82^{a}	1710.50±673.80^{ab}

In the same line, for each year, parameter and rootstock studied, mean values with different letters differ significantly ($p < 0.05$).

Chemical characteristics of the rootstocks

Antioxidant activity. The results obtained for the EC_{50} values of the DPPH and ABTS radicals are shown in Table 3. In our test conditions, GN showed the highest antioxidant activity in the DPPH test with an EC_{50} of 0.28 mg mL^{-1} versus 0.34 mg mL^{-1} obtained for RP-R and 0.44 mg mL^{-1} of GF 677 (Table 3). For both methods tested GN reported significantly higher antioxidant activity comparatively with the other two tested rootstocks. In contrast RP-R leaves were less antioxidant in the ABTS method and GF 677 the lowest antioxidant in the DPPH assay.

To study the reducing power, we determined the EC_{50} as the concentration at which we obtained an absorbance of 0.5 at a wavelength of 700 mn. For GN we obtained the EC_{50} for a concentration of 0.75 mg mL^{-1} whereas to obtain this same reading in the RP-R and GF 677 rootstocks we had to increase the concentrations to 1.10 mg mL^{-1} and 1.31 mg mL^{-1} respectively (Table 3).

Table 3

Antioxidant capacity of the leaves of different rootstocks under study through DPPH and ABTS scavenging assays (n = 9; mean value ± standard deviation)

Rootstock leaves	*EC_{50} ABTS (mg mL^{-1})*	*EC_{50} DPPH (mg mL^{-1})*	*EC_{50} reducing power (mg mL^{-1})*
GF 677	0.44 ± 0.01^{b}	0.41 ± 0.07^{c}	1.31 ± 0.02^{c}
GN	0.39 ± 0.01^{a}	0.28 ± 0.02^{a}	0.75 ± 0.01^{a}
RP-R	0.50 ± 0.04^{c}	0.34 ± 0.07^{b}	1.10 ± 0.02^{b}

In the same column, for each parameter and rootstock studied, mean values with different letters differ significantly ($p < 0.05$).

Determination of different phenolic groups. The RP-R and GN showed almost identical contents in hydroxycinnamic acid derivatives, around 185.00 mg of CAE g^{-1}. Significant differences were found when comparing the hydroxycinnamic acid derivatives contents of these two rootstocks with GF 677, which was found to have 40% less hydroxycinnamic acid derivatives in its composition (Table 4). This same trend was repeated when measuring the simple phenols of the rootstocks under test. Again, GF 677 stood out for its lower content of simple phenols: 258.93 mg GAE g^{-1} compared to 289.21 mg GAE g^{-1} obtained for GN or 306.10 mg GAE g^{-1} of RP- R. The GN samples exhibited statistically higher flavonoid contents than those of RP-R and GF 677. It should be noted that the differences were especially notable between the GN and GF 677 rootstocks, while the concentration of flavonoids obtained for GF 677 revolves around 61.66 mg QE g^{-1}, GN reported 70% higher values with contents in flavonoids that exceed 100.00 mg QE g^{-1}.

Table 4

Phenolic composition of the leaves of the different rootstocks under study (n = 9; mean value ± standard deviation)

Rootstock leaves	*Phenolic acids (mg cafeic acid / g extract)*	*Simple phenols (mg galic acid/ g extract)*	*Flavonoids (mg quercetin / g extract)*
GF 677	130.31 ± 19.83^{a}	258.93 ± 28.43^{a}	61.66 ± 8.14^{a}
GN	184.14 ± 10.99^{b}	289.21 ± 40.54^{b}	105.82 ± 16.90^{c}
RP-R	185.57 ± 37.68^{b}	306.10 ± 13.68^{b}	91.75 ± 10.00^{b}

In the same column, for each parameter and rootstock studied, mean values with different letters differ significantly ($p < 0.05$).

Contribution of biostimulants to the plant development. As described in Table 5, the biostimulants contribution during the first year of the young trees promoted the development of their aerial part. Trees treated with biostimulants 1 and 2 showed greater vigor than the control trees for the three rootstocks under study. Broadly, trees treated

with biostimulant 2 were taller (97.76 cm) and the growth of young shoots compared to biostimulant 1 was accelerated, except for the GN rootstock where no differences were observed in the use of one or the other biostimulant in terms of height.

There were also statistically significant differences in the trunk cross-sectional area attributable to the contribution of biostimulants in all the rootstocks. Both biostimulants caused a thickening of the trunk cross-sectional area, but the application of biostimulant 2 proved a higher thickening. Compared to the control trees, the thickening of the trunk cross-sectional area produced by biostimulant 2 was of 35.00% in GF, 677.59% in GN and 67.00% in RP-R.

Fertilization had also impact on the radicular development of the rootstocks under study during the trial's first year. Both biostimulants promoted root growth so that the treated trees presented a greater number of both main and secondary roots than the control trees. We counted an average of 7 main roots in RP-R control samples while for biostimulant 1 and 2 the average number of main roots was 13 and 10 respectively. The inverse relationship that was observed in the control trees between the number of main and secondary roots and their diameter (Table 2) disappears when biostimulants are applied being impossible to define in our essay a clear relation of the effect of the fertilization in the individual diameter of the roots. The architecture of the roots was partly modified by the contribution of biostimulants in comparison with control trees. All the individuals treated with one or the other biostimulant increased the distance between the main root and its first bifurcation to secondary root. While distance between the main root and its first bifurcation in GF 677 control samples was 1.32 mm the contribution of biostimulant 1 and 2 increased it to 8.92 mm and 4.94 mm respectively. Although this phenomenon was not observed in the bifurcations of secondary to tertiary roots. The contribution of biostimulant 2 also produced radicular systems whose depth exploration (measured through the maximum length of roots) was inferior to control trees, for example GN samples treated with biostimulant 2 were 200 mm shorter than their control. Considering the radicular biomass, the use of the biostimulant 2 originated an increase in the weight of the root system in all the rootstocks under study (26.44% for RP-R, 16.93% for GF 677 and 48% for GN) while the biostimulant 1 only produced increases in the weight of roots of GF 677 and GN (36.93% and 48.16% respectively).

Although during the first year of tree growth statistically significant differences were observed between the samples subjected to a contribution of biostimulants and the control samples, during the second year these differences are extremely attenuated. The contribution of biostimulants only promoted a greater vegetative development in the GF 677 rootstock (171.75 cm for biostimulant 1 and 148.90 cm for biostimulant 2 compared to control trees with and average height of 108 cm) with hardly any differences depending on the type of treatment received in the RP-R and GN rootstocks. Both biostimulant 1 and 2 produced increases in the trunk cross-sectional area of RP-R (24.49 mm and 29.52 mm respectively) and GF 677 (27.08 mm and 28.13 mm respectively) but no difference was observed between the control and the GN individuals. Regarding the root system, as occurred in the one-year samples, a relationship between the contribution of biostimulants and the increase in the number of both main (for all the rootstocks) and secondary roots (for RP-R and GF 677) can be observed. Biostimulant 2 led to a greater weight of the radicular system again in the individuals of RP-R (283.04 g) and GF 677 (285.60 g) but not in the GN rootstocks. We observed that there is a certain relationship between the effect of the contribution with biostimulants and the vigor of the rootstocks so that from the second year the effect of the biostimulants hardly produce light changes in the GN (vigorous rootstock of the trial). Probably when it comes to pot trials, the roots have colonized all the available space, which is why this same trial will be established in the future in an open field.

Table 5

Effects of the different biostimulants on the rootstock development during the two year-study (n = 10; mean value ± standard deviation)

Year of study	*Year 1*								
Rootstock	*RP-R*			*GF 677*			*GN*		
	Biost. 1	*Biost. 2*	*Control*	*Biost. 1*	*Biost. 2*	*Control*	*Biost. 1*	*Biost. 2*	*Control*
Vegetative system									
Tree height (cm)	80.00±3.16^{b}	97.76±0.46^{c}	57.60±1.14^{a}	98.30±0.62^{b}	111.92±0.72^{c}	46.80±0.83^{a}	121.54±0.78^{b}	121.00±1.57^{b}	61.00±0.70^{a}
Trunk longitude (cm)	41.66±3.65^{b}	30.54±0.45^{a}	31.40±7.95^{a}	32.62±0.39^{c}	27.80±2.42^{b}	22.70±0.46^{a}	35.02±0.16^{c}	33.94±0.78^{b}	24.28±0.33^{a}
Young shoots weight (g)	20.14±1.04^{b}	24.23±1.56^{c}	8.39±1.01^{a}	24.11±0.19^{b}	52.20±5.39^{c}	8.04±0.07^{a}	32.43±0.25^{b}	80.62±7.77^{c}	9.32±0.05^{a}
Radicular system									
Diameter of grafted area (mm)	9.44±0.06^{b}	13.00±0.08^{c}	7.72±0.68^{a}	9.85±0.10^{b}	10.77±0.10^{c}	7.95±0.14^{a}	11.29±0.27^{b}	14.08±0.26^{c}	8.81±0.13^{a}
Number of tap roots	13.00±1.64^{c}	10.00±0.89^{b}	7.00±1.30^{a}	7.00±6.89^{a}	13.00±2.40^{c}	8.00±0.54^{b}	7.40±0.54^{a}	9.80±2.28^{b}	7.60±0.54^{a}
Tap root diameter (mm)	3.39±0.35^{a}	3.88±0.06^{b}	5.31±0.02^{c}	2.52±0.13^{a}	3.09±1.01^{a}	2.81±0.06^{a}	4.70±0.15^{b}	4.79±0.12^{b}	3.82±0.05^{a}
Distance of tap roots to first bifurcation (mm)	4.02±0.99^{c}	2.88±0.34^{b}	1.71±0.72^{a}	8.92±0.04^{c}	4.94±0.30^{b}	1.32±0.02^{a}	2.28±0.12^{b}	2.74±0.42^{c}	1.24±0.01^{a}
Tap root weight (g)	11.69±0.44^{a}	22.91±1.11^{b}	10.75±0.85^{a}	13.19±0.08^{b}	17.59±1.54^{c}	8.37±0.23^{a}	21.36±0.11^{b}	29.47±0.76^{c}	13.30±0.25^{a}
Number of lateral roots	57.00±13.24^{a}	88.00±1.58^{b}	50.40±3.04^{a}	17.00±1.09^{c}	13.00±2.40^{b}	8.00±0.54^{a}	55.00±1.00^{c}	49.00±5.54^{b}	35.00±1.92^{a}
Lateral root diameter (mm)	0.44±0.09^{a}	0.79±0.03^{b}	0.63±0.29^{ab}	2.52±0.13^{a}	3.09±1.01^{a}	2.82±0.05^{a}	1.08±0.03^{b}	1.31±0.16^{c}	0.81±0.03^{a}
Distance of tap lateral roots to first bifurcation (mm)	2.55±0.25^{a}	3.44±0.23^{b}	4.34±0.96^{c}	5.85±0.07^{b}	6.07±1.97^{b}	4.48±0.02^{a}	3.91±0.04^{b}	3.63±0.27^{a}	5.84±0.03^{c}
Lateral root weight (g)	4.76±3.37^{a}	7.92±0.24^{b}	5.23±0.78^{a}	12.14±0.27^{b}	11.87±2.50^{b}	7.43±0.19^{a}	14.90±0.07^{b}	18.22±3.40^{c}	6.58±0.08^{a}
Absorbing root weight (g)	2.53±0.32^{a}	3.92±0.70^{b}	4.04±0.55^{b}	1.03±0.78^{a}	2.18±0.12^{b}	1.23±0.16^{a}	2.46±0.08^{a}	4.72±0.93^{b}	3.01±0.05^{a}
Total roots dry weight (g)	10.23±2.06^{a}	14.20±2.30^{b}	11.23±1.08^{a}	14.31±0.54^{c}	12.22±1.19^{b}	10.45±0.54^{a}	20.61±0.42^{b}	20.59±2.38^{b}	13.91±0.63^{a}
Roots longitude (mm)	750.60±20.00^{a}	773.40±13.10^{a}	920.00±73.10^{b}	727.40±5.10^{a}	701.00±13.88^{a}	758.00±8.30^{a}	886.60±4.10^{c}	562.20±39.00^{a}	762.00±16.40^{b}
Root to Shoot ratio	0.50±0.08^{a}	0.58±0.11^{a}	1.33±0.32^{b}	0.59±0.10^{b}	0.23±0.09^{a}	1.29±0.45^{c}	0.63±0.09^{b}	0.25±0.02^{a}	1.49±0.34^{c}
Year of study	*Year 2*								
Rootstock	*RP-R*			*GF 677*			*GN*		
	Biost. 1	*Biost. 2*	*Control*	*Biost. 1*	*Biost. 2*	*Control*	*Biost. 1*	*Biost. 2*	*Control*
Vegetative system									
Tree height (cm)	115.20±1.16^{a}	129.04±1.41^{b}	135.93±1.71^{c}	171.75±11.66^{b}	148.90±26.16^{b}	108.00±4.24^{a}	145.50±1.62^{a}	158.55±23.40^{a}	147.40±32.60^{a}
Trunk longitude (cm)	44.99±3.95^{a}	43.97±0.64^{a}	46.78±11.85^{a}	36.40±7.91^{a}	45.05±11.95^{a}	37.95±21.28^{a}	29.35±6.57^{a}	44.1±6.92^{a}	32.15±20.94^{a}
Young shoots weight (g)	212.31±10.96^{c}	51.85±3.35^{a}	101.79±12.35^{b}	387.16±42.33^{b}	139.10±85.70^{a}	73.20±41.15^{a}	210.72±82.84^{a}	145.85±94.25^{a}	137.45±15.34^{a}
Radicular system									
Diameter of grafted area (mm)	25.49±0.15^{b}	29.52±0.18^{c}	23.55±2.08^{a}	27.08±0.82^{b}	28.13±0.79^{b}	22.02±0.74^{a}	30.12±1.61^{a}	28.46±2.65^{a}	29.17±1.09^{a}
Number of tap roots	68.8±2.94^{c}	61.6±5.36^{b}	23.8±4.56^{a}	65.00±33.94^{ab}	86.00±16.26^{b}	34.00±4.24^{a}	56.00±8.48^{b}	55.00±4.94^{b}	22.00±3.53^{a}
Tap root diameter (mm)	3.52±0.36^{a}	3.83±0.34^{a}	7.22±0.03^{b}	3.37±1.01^{a}	2.49±0.60^{a}	2.99±1.70^{a}	4.16±0.23^{a}	3.42±0.90^{a}	5.86±1.09^{a}
Distance of tap roots to first bifurcation (mm)	8.84±2.17^{b}	8.55±1.01^{b}	13.07±5.52^{a}	13.70±3.59^{a}	12.52±4.87^{a}	10.88±5.49^{a}	10.99±5.23^{a}	10.31±1.40^{a}	8.62±0.32^{a}
Tap root weight (g)	115.86±4.35^{a}	226.60±10.90^{c}	180.70±14.28^{b}	146.35±48.15^{b}	195.55±54.37^{b}	97.45±0.01^{a}	197.40±66.18^{a}	270.25±92.27^{a}	249.75±139.37^{a}
Number of lateral roots	413.00±109.95^{a}	697.00±11.66^{b}	450.00±27.23^{a}	284.50±54.44^{a}	501.50±146.37^{b}	304.50±96.37^{a}	311.00±54.24^{a}	356.00±36.06^{a}	287.00±141.40^{a}
Lateral root diameter (mm)	0.47±0.10^{a}	0.67±0.02^{a}	0.65±0.30^{a}	1.07±0.25^{a}	0.91±0.15^{a}	0.95±0.02^{a}	1.26±0.26^{a}	1.17±0.28^{a}	1.26±0.17^{a}
Distance of tap lateral roots to first bifurcation (mm)	8.60±0.86^{a}	12.49±0.85^{b}	13.99±3.09^{b}	15.64±5.16^{a}	19.77±8.73^{a}	18.05±5.06^{a}	17.25±3.15^{a}	15.5±2.08^{a}	15.21±0.75^{a}
Lateral root weight (g)	28.46±20.12^{a}	44.05±1.35^{b}	58.57±8.74^{c}	73.4±38.18^{a}	90.05±28.63^{a}	55.15±9.68^{a}	197.40±66.18^{a}	270.25±92.27^{a}	249.75±139.37^{a}
Absorbing root weight (g)	9.03±1.16^{a}	7.92±1.41^{a}	12.38±1.71^{b}	8.85±2.05^{a}	9.55±1.20^{a}	6.32±4.27^{a}	3.65±2.75^{a}	4.40±2.54^{a}	6.75±1.90^{a}
Total roots dry weight (g)	147.25±29.70^{a}	283.04±45.98^{c}	232.36±22.38^{b}	217.40±89.66^{b}	285.6±83.01^{b}	152.6±3.67^{a}	285.25±56.78^{a}	352.60±99.98^{a}	351.40±197.99^{a}
Roots longitude (mm)	1103.70±29.21^{a}	1554.50±264.00^{b}	1720.40±136.70^{b}	1163.00±322.40^{a}	1124.50±221.30^{a}	1142.00±282.28^{a}	1199.50±284.90^{a}	1421.50±27.64^{a}	1710.50±673.80^{a}
Root to Shoot ratio	0.69±0.10^{a}	5.45±1.22^{c}	2.28±0.79^{b}	0.56±0.24^{a}	2.05±0.73^{b}	2.08±0.88^{b}	1.35±0.27^{a}	2.41±0.89^{b}	2.55±0.72^{b}

In the same line, for each year, parameter and rootstock studied, mean values with different letters differ significantly ($p < 0.05$).

Discussion. As in other crops, in the almond tree, high quality seedlings are sought when establishing new plantations with a good development both aerial and radicular.

The use of rootstocks is a tool widely used in agriculture for its ability to modify the size and shape of grafted varieties by altering the distance between knots and the angle of the branches and modifying the rates of active growth (Inglese et al 2002). In this study, GN stood out for its greater vegetative vigor in both the one-year and two-year samples compared to GF 677 and RP-R. These results agree with those obtained by Felipe (2009) that described GN as a very vigorous rootstock capable of provoking a greater volume of biomass by producing a greater number of young shoots, as occurs in our study (Table 2). As in the study by Mondragón-Valero et al (2017) the greater vigor of GN translates into a larger trunk diameter. A larger trunk cross-sectional area can induce or modify the height of the tree, the volume of the crown, the structure of the branches and the productivity and size of the fruits (Srivastava et al 2017). Several authors describe a negative relationship between the vigor of the rootstock and the productivity of the cultivar in *Prunus* species (Marra et al 2013) but nevertheless positive with the accumulated yield (Reig et al 2016). It should be considered that the rootstocks capable of inducing a certain vigor can also modify the expression of certain genes, as in the case of the cherry tree, the genes related to the metabolism of the flavonoids and the synthesis of the cell wall (Prassinos et al 2009). On the other hand, the ability of a plant to produce different types of roots is an aspect of its plasticity that has important characteristics of adaptation. As in other crops, the number of roots and their distribution varies depending on the genotype of the individual under study highlighting the number of roots and the architecture of the root system as very important factors in tree stability (Dupuy et al 2007). In our characterization, the rootstock that had the highest number of main roots was GF 677; however, it was RP-R that stood out for its higher number of secondary roots. The ramification of the root system through the formation of secondary or lateral roots represents an essential element in the adaptation of the system to its environment and is regulated by hormonal and nutritional signals that act locally to induce or inhibit the proliferation of roots (Bellini et al 2014). Thanks to these adaptive responses the plant can increase the contact surface with the soil for a greater capture of resources (Atkinson et al 2014).

Another of the cultivation practices that most influences the quality of nursery seedlings is fertilization. The contribution of nutrients in the first stages of the plant development is a key factor, especially for seedlings produced in containers in which the limited volume seriously hinders growth. Fertilization affects both vegetative and root growth of plants, improves rooting and growth capacity after transplantation, and increases resistance to water stress, low temperatures and certain diseases (Grossnickle 2000). In addition, the mobilization of internal reserves provides the seedlings with some independence from the external availability of nutrients (Cherbuy et al 2001). These properties are essential for early establishment in the open field, especially when unfavorable conditions take place. In our study, the use of both biostimulants promoted the development of the aerial part of the rootstocks especially during the first year. Similar results were obtained by Saa et al (2015) when applying foliar biostimulants from different origins in a one-year-old almond tree plantation.

Biostimulant 2 based on humic and fulvic substances promoted more actively the vegetative development of all the rootstocks under study. The contribution of humic substances propitiated the vegetative growth in the tests carried out by Fathy et al (2010) in apricot trees and by Laila et al (2013) in olive trees. The investigations of Zandonadi et al (2010) conclude that humic substances induce the growth of plants through the activation of the plasma membrane. Similarly, the proliferation of shoots by humic substances may be caused by the exogenously applied acids obtaining increases of 22% of the dry weight of biomass in different species (Rose et al 2014). When comparing our results with the latter, the obtained increases in biomass produced by biostimulant 2 are much more significant, which would be explained by the high variability of the effect of humic acids depending on the origin of the material, the species treated, the way and dose of application and the environmental conditions to which the crop is subjected but

also by the possible synergistic action of the rest of the compounds of biostimulant 2 (Yakhin et al 2017).

Although to a lesser extent, the biostimulant 1 also produced an increase in the vegetative development of the samples of one year. Halpern et al (2015) showed that the contributions of hydrolyzed proteins can promote vegetative growth and the absorption of macro and micronutrients, resulting in an increase in crop productivity. This increase in aerial biomass can be attributed to an increase in the foliar nitrogen content that causes a better photosynthesis process and promotes the translocation of the synthesizers to the sinks (Colla et al 2017). Amino acids and small peptides are absorbed by both leaves and roots and are then translocated to the rest of the plant. However, the availability of amino acids for root absorption can be strongly diminished by the action of soil microorganisms. Although absorption depends, among others, on soil conditions and on the type of amino acid and its concentration, it is estimated that only between 6 and 25% of the amino acids supplied externally via the root system go to the roots (Moe 2013). This fact could explain the lower incidence of biostimulant 1 in the development of the rootstocks under study.

The contribution of biostimulants also led to an increase in root biomass both in weight and number of roots in one-year-old individuals. The contribution of both biostimulants was able to increase the weight of the radical systems of GF 677 and GN although only biostimulant 2 achieved this effect in the RP-R rootstock. Several experimental studies in both controlled and open-field conditions have shown the relationship between the supply of hydrolyzed proteins and the development of the biomass of the root system (Halpern et al 2015; Colla et al 2017). Some studies indicate that glutamate receptors in plants can be activated by other amino acids besides glutamate and that can mediate a series of plant responses such as changes in the architecture of the root (in our case increased the distance between the main root and the secondary root), in the metabolism of carbon and in photosynthesis (Forde & Roberts 2014). In line with these investigations, several studies have contrasted the effect of humic and fulvic acids as promoters of root development causing greater biomass and inducing and increasing the formation of lateral roots. (Rose et al 2014). The fact that the roots are in constant growth and renewal can suppose a mechanism of defense of the plants to overcome different types of stress (Amador et al 2012), hence biostimulants based on amino acids (Colla et al 2017) as on humic and fulvic acids (Nardi et al 2016) can be considered as mitigators of the stress response.

Regarding the antioxidant power of almond rootstocks, it should be noted that natural antioxidants are produced as complex mixtures of compounds that react differently to different radicals, hence the analyses of antioxidant capacity may vary depending on the type of test carried out. In our case, coinciding with the results obtained by Floegel et al (2011), the ABTS assays obtained higher levels of antioxidant capacity for all concentrations and rootstocks than the DPPH assays. The advantage of the ABTS radical is its high reactivity and, therefore, its ability to react against a wider range of antioxidants (Mareček et al 2017). The results suggest that the ABTS assay better reflects the antioxidant content of the almond rootstocks than the DPPH assay although, as reported by Dudonné et al (2009) in their studies, in our case there is also a strong positive correlation between both tests. The leaves of all the rootstocks under study showed high percentages of inhibition for both tests, being the antioxidant capacity of the almond leaf much higher than the olive leaf (Benavente-García et al 2010) but lower to walnut and chestnut leaves (Pereira et al 2007; Barreira et al 2008).

As in other previous studies related to almond plant material (Sfahlan et al 2009) in this research we found significant differences in the reducing power of the samples that derive from the different genotypes studied. In general, the three rootstocks showed a high capacity of reducing power with EC_{50} that did never exceed 1.31 mg mL^{-1}. Both the leaves of GN, RP-R and GF 677 showed a greater reducing power than moringa leaves (Iqbal & Bhanger 2006) but lower than cacao leaves (Osman et al 2004).

Phenolic compounds are attributed multiple biological properties such as antioxidant activity since they can act both by sequestering free radicals and preventing their formation (Boulanouar et al 2013). Several previous studies highlight the

antioxidant potential of phenols in other plant species (Pereira et al 2007; Ferreira et al 2007). In the case of almond leaves this relationship is verified, we observe that the extracts with the highest content of phenols have the lowest EC_{50}, highlighting again the GN rootstock above the rest. The phenol content of almond leaves is much higher than that of apple leaves (Mayr et al 1995), walnut leaves (Miliauskas et al 2004) or a worldwide known source of antioxidants such as green tea leaves (Arcan & Yemenicioğlu 2009).

The high phenolic content of the almond leaves can influence the defense mechanisms of the plant. The contribution of phenols in resistance to plant diseases is largely based on their cytotoxicity (Iqbal et al 2015; Wink 2017). Puupponen-Pimia et al (2001) located different phenolic compounds extracted from raspberry and mulberry that showed antimicrobial activity against two strains of *Escherichia coli* and one strain of *Salmonella enterica*. In a study on almond leaf scorch produced by *Xylella fastidiosa* in different almond cultivars, Wilhelm et al (2011) concluded that the higher phenolic concentration in the xylem fluid of cultivars resistant to this pathology could decrease the survival of the bacteria during winter. In another work with almond hybrids Misirli et al (2001) found that leaf samples with a higher quercetin content were more resistant to inoculation of *Pseudomonas amygdali*. In our case, GN stands out against RP-R or GF 677 because of its higher flavonoid content measured in mg of quercetin per gram of extract. Tattini et al (2006) allude to a close relationship between tolerance to oxidative stress and the accumulation of flavonoids. The leaves of GN draw attention because of their reddish appearance that comes from their high concentration in anthocyanins (Zrig et al 2011). Among the flavonoids, anthocyanins are highly water-soluble pigments derived from the precursors of flavonoids through the shikimic acid pathway. They protect chloroplasts from senescent leaves shadow adapted leaves from photooxidative stress produced by prolonged exposure to high solar radiation (Gould et al 2002). Since anthocyanins are osmotically active, their concentration at high levels can increase the resistance of plants to certain stresses thanks to greater osmotic control (Manetas 2006). In addition, it has been shown that leaves with high anthocyanin levels have greater antioxidant capacity than green leaves and that anthocyanins contribute to this capacity more than other low molecular weight compounds (Gould et al 2002). Zrig et al (2011) concluded that the content of anthocyanins plays an important physiological role in the protection of almond rootstocks against salinity. The GN rootstock was able to overcome saline toxicity thanks to the use of anthocyanins that abound in its leaves, while GF 677 showed a good response to saline stress due to the high content of carotenoids.

There are many recent studies in Prunus (Giorgi et al 2005; Drogoudi & Tsipouridis 2007; Jakobek et al 2009) but also in other genus (Hudina et al 2014; Kviklys et al 2014) demonstrating that the antioxidant activity of the grafted varieties varies according to the rootstock selection. Of special interest is the research of Satisha et al (2008) concluding that rootstocks may influence the biochemical composition of the scion leaves grafted onto them, which in turn affects the degree of resistance or susceptibility to powdery mildew disease in grapes.

Conclusions. The GN rootstock stands out against GF 677 and RP-R for a greater vigor of the vegetative system with greater weight of pruning and greater trunk cross-sectional area. Moreover, this rootstock presents values in weight of the radicular system superior to the other rootstocks, especially in the samples of two years. RP-P that stands out for its greater number of secondary roots that allow it to increase the contact surface with the soil for a greater capture of resources and a better adaptation to the environment, this rootstock also shows the maximum length of the radical system that translates into a higher level of depth exploration.

Regarding the chemical characterization, we observed that broadly the almond leaves have a great antioxidant power, although differences are observed depending on the rootstock tested. GN presented the greatest antioxidant power and the higher phenol content of the rootstocks under study. These chemical properties are important as they can influence the defense mechanisms of the plant by inducing resistance to certain stresses, pathogens or diseases.

The fertilization with biostimulants had a significant impact on the development of the plants both at the aerial and radicular levels and was able to short the nursery deadlines. Although the results of biostimulant 1 were higher, both biostimulants favored the proliferation of primary and/or secondary roots, achieving plants with better or faster adaptation capacity to the orchard. Roots in constant growth and renewal suppose a mechanism of defense for the plants, hence the two biostimulants under study can be considered as mitigators of the response to different stresses. Biostimulants must be considered an important tool that allows a faster and better adaptation of almond crops within the framework of sustainable agriculture.

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Conflicts of interest. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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