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Inhibitory effect of jasmonic acid and ethylene on epicotyl growth and bud induction in the maritime pine, *Pinus pinaster* Soland. in Ait

Maria Teresa Martin[2*], Hilda Pedranzani[3], Patricia García-Molinero[2], Valentin Pando[4] and Rosario Sierra-de-Grado[1]

1. Departamento de Producción Vegetal y Recursos Forestales, Escuela Técnica Superior de Ingenierías Agrarias, Universidad de Valladolid, Avda. de Madrid, 44; Palencia E-34007 Spain.
2. Departamento de Viticultura, Instituto Tecnológico Agrario de Castilla y León, Ctra. Burgos, Km. 119; Valladolid E-47071. ESPAÑA.
3. Laboratorio de Fisiología Vegetal, Departamento de Ciencias Agropecuarias. FICES Universidad Nacional de San Luis, Avda. 25 de Mayo 384, Villa Mercedes, San Luis, Argentina.
4. Departamento de Estadística e Investigación Operativa. Escuela Técnica Superior Ingenierías Agrarias. Universidad de Valladolid. Avda. de Madrid 44; Palencia E-34007 Spain.

Key words: micropropagation, phytohormones, plant growth regulators, 6-benzylaminopurine, *in vitro* plant culture.

ABSTRACT: Two independent parameters, epicotyl height (cm) and number of induced buds were studied on *Pinus pinaster* explants to analyse the effects of three phytohormones (6-benzylaminopurine, jasmonic acid, ethylene) which were combined or not in 11 different treatments. Epicotyle length diminished significantly in relation to the control medium (medium without exogen phytohormones) in presence of jasmonic acid, 6-benzylaminopurine or Ethephon (which is converted to ethylene in plants) in any of treatments. Concentrations of 100 µM of jasmonic acid and Ethephon had a greater inhibitory effect than the treatments with 10 µM. In addition to that, jasmonic acid was a stronger inhibitor than Ethephon in any of the tried combinations. There were no significant differences between the control treatment and the treatments with only 10 µM of jasmonic acid or Ethephon. However, 10 µM 6-benzylaminopurine induced bud formation. The different combinations of 6-benzylaminopurine with jasmonic acid and Ethephon showed that concentrations of 10 to 100 µM did not affect the number of induced buds. Jasmonic acid had an inhibitory effect which Ethephon only showed when combined with 100 µM of jasmonic acid and 10 µM of 6-benzylaminopurine. Three response groups were defined by cluster analysis: group 1 produced the greatest mean number of buds (4 to 5) and a mean epicotyl growth of 1 to 1.5 cm; group 2 produced 2 to 4 buds and a mean growth of 0.5 to 1.2 cm; group 3 produced only one bud and a mean epicotyl length of 1.2 to 2 cm.

Introduction

In most species, once the programmes for genetic improvement are advanced and outstanding individuals are available, one may resort to cloning of the selected genotypes to obtain the greatest possible descent, particularly if the improved trait does not present high heritability. The maritime pine, *Pinus pinaster* does not spontaneously spread through vegetative propagation. Propagation by cuttings or plantlets has been tried with reduced success. *In vitro* culture is introduced as an alternative that could generate an important number of copies of the same genotype (Tang *et al*., 2006). However, like other types of vegetative propagation, *in vitro* culture of conifers is not easy, and becomes more difficult as the individual to be cloned gets older (Abdullah *et al*., 1987). Another aspect that conditions the experiment is the influence of the genotype; there

*Address correspondence to: Maria Teresa Martin.
E-mail: *marvilte@itacyl.es*
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are genotypes which are more successful than others (Tang *et al*., 2001). *In vitro* culture has the additional advantage of allowing the conservation of a great number of genotypes during a theoretically unlimited period of time in a reduced space through the establishment of a bank of *in vitro* germplasm. As the first step for clone propagation of outstanding individuals of *Pinus pinaster*, the *in vitro* technique has been refined by using young material, i.e., newly germinated plantlets.

To our knowledge this is the first studies that have been carried out with *Pinus pinaster*, on the effect of phytohormones of maturity on *in vitro* bud induction and growth. Jasmonic acid and Ethephon (2-chloroethyl phosphonic acid, a plant growth regulator which is converted to ethylene by plant metabolism) have been tested alone or in a different combinations with the 6-benzylaminopurine cytokinin. The latter is generally used as a phytohormone in the micropropagation of plants to activate dormant lateral buds, induce cell division and shoot formation and delay senescence. Ethylene participates in the regulation of a large variety of developmental processes, from seed germination to cell elongation, fruit ripening, organ senescence and abscission (Stepanova and Alonso, 2005). Jasmonic acid is usually present in the picomolar range per gram of fresh leaf tissue, and can quickly increase under external stimuli. Some organs and tissues exhibit more than 10 times the level found in leaves, which suggests that these high levels have different functions in the regulation of particular processes of development (Wasternack and Hause, 2002).

Jasmonates are signal compounds that appear not only as a defence against infections, and to promote tolerance to salinity and drought, but also in a variety of physiological mechanisms related to biotic and abiotic stress (Creelmann and Mullet, 1995, 1997; Davis *et al*., 2002; Pedranzani *et al*., 2003). Thus, we studied the individual and combined effects of the three phytohormones: 6-benzylaminopurine, jasmonic acid and ethylene.

Jasmonic acid and ethylene are phytohormones associated with stress, and they are involved in the regulation of the expression of genes intervening in maturity and senescence (Bohnert *et al*., 1995; Park *et al*., 1994). It has been recently shown that jasmonic acid is involved in the response of *Pinus pinaster* to stress due to cold and drought, and that its phenotypic expression is probably related to Spanish genotypes (Pedranzani *et al*., 2007). A number of positive and negative interactions between ethylene and jasmonate have been described both at physiological and molecular levels (Ellis and Turner, 2001). Both hormones participate in the defence response to a variety of fungal pathogens. Similarly, both hormones positively interact on the transcription of several wounding-regulated genes and on the formation of the apical hook (Rojo *et al*., 2003). Analysis of the regulation of gene expression in response to both ethylene and jasmonic acid in different genetic backgrounds has uncovered one of the best-characterized examples of interactions between hormones at the molecular level (Lorenzo *et al*., 2003).

In the current paper, two independent parameters, epicotyl growth (cm) and number of induced buds were studied on *Pinus pinaster* explants to analyse the effects of three phytohormones combined in 11 different treatments.

Material and Methods

Material

Commercial seeds of *Pinus pinaster* Soland. in Ait. from Sierra de Guadarrama, were provided by El Serranillo, gathered in 1998/99 and were from different progenies. The three phytohormones, Ethephon (2-chloroethylphosphonic acid), jasmonic acid and 6-benzylaminopurine were acquired from Merck and Sigma. The phytohormones were added to the culture media after autoclaving.

Sterilization of seeds

Pine seeds were immersed in water for 12 hours. Those that did not float were surface sterilized with 70% ethanol for 2 min., then washed in sterile water for 15-20 min., then in calcium hypochlorite at 3%, for 15-20 min. and finally three times in sterile water for 15-20 min.

Germination

Once sterilized, 20 seeds were placed on moist sterile vermiculite in jars of 10 cm in diameter by 10 cm in height. The seeds were incubated at 22ºC, with a relative humidity of 75-85%, and a photoperiod of 16 light: 8 hours darkness. After the pericarp was loose, the cotyledons sprouted and the radicle emerged, the seedlings were surface sterilised with calcium hypochlorite at 1%, before sowing them in the culture medium.

In vitro setting

Seedlings used as explants had the radicle cut, and only a 0.5 cm end left. This portion was introduced in the culture medium.

Bud induction and growth

The medium used to cultivate buds was Campbell and Durzan (1975) macronutrients with Murashige and Skoog (1962) micronutrients, with vitamins (George, 1993). Iron (80 mg/L, sucrose (20 g/L), and agar (8 g/L) were added. Three phytohormones (6-benzylaminopurine, jasmonic acid and Ethephon) were added to the basic medium in 11 different combinations (Figs. 2 and 3). Both the number of induced buds and growth (epicotyl height, apical needles included, in cm) were registered for each treatment after 23 days in culture.

Statistical analysis

Differences in the mean number of buds and growth in the 11 treatments were analyzed by one way ANOVA. The Tukey test was used for post hoc comparisons between pairs. The effects of the different hormones (6-benzylaminopurine, jasmonic acid and Ethephon) and the quadratic effects of jasmonic acid and Ethephon were analyzed by regression analysis. A general GLM model was performed for height and number of buds. In addition, a generalised GLZ model for number of buds was performed, due to the lack of normality in this variable. This model was consistent with the GLM model, thus only the GLM model is presented here. All these analyses were performed with STATISTICA. The relationship between the active variables (epicotyl height and bud number) was tested by main components analysis (SPAD programme).

Results

Main components analysis for the active variables, epicotyl growth (cm) and number of buds, yielded an angle of about 90º (Fig. 1), thus showing that they were unrelated variables.

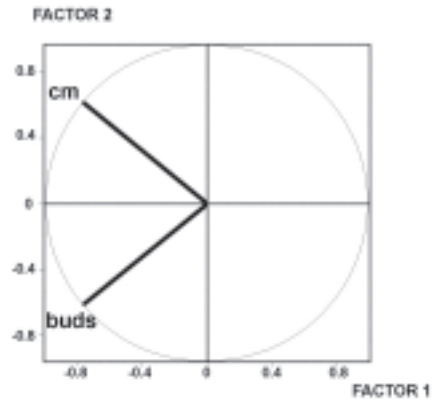

FIGURE 1. SPAD analysis of the main components for epicotyl height and number of induced buds showing no interrelation between these variables (the angle is near 90º).

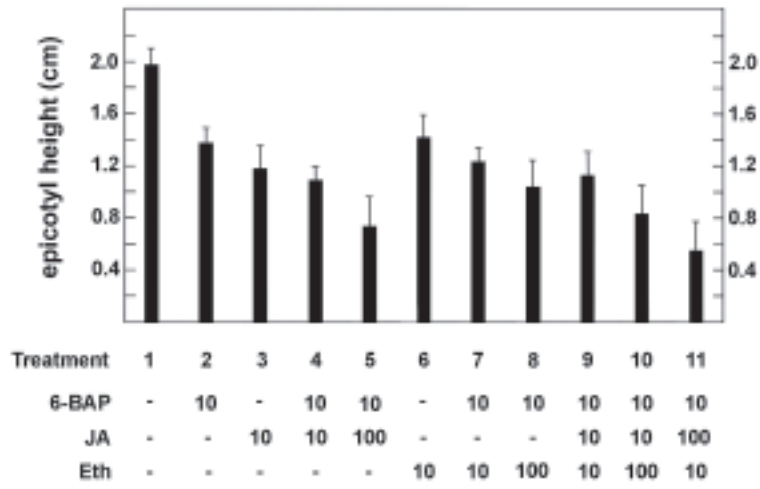

FIGURE 2. Mean values for epicotyl growth (cm) in the different treatments. Data are the mean ± standard deviations.

Epicotyl growth

In figure 2, the mean values for epicotyl height in each of the 11 treatments are presented; for each treatment error bars indicate the standard deviation of the replicates. ANOVA test indicated statistically significant differences ($P < 0.0001$). Significant differences between pairs are indicated in Table 1. The GLM model for regression analysis was significant ($P < 0.0001$) and explained 31% of the variability. The equation obtained was Height = 1.7592 – 0.0332*BA – 0.0374*E + 0.0003*JA^2 + 0.0002*E^2, where BA is the effect attributed to 6-benzylaminopurine, JA is that of jasmonic acid and E is that of Ethephon all the single and quadratic effects were significant. Therefore, the phytohormones had an inhibitory effect on epicotyl growth.

In the media without phytohormones (treatment 1, control), epicotyl height reached a mean 1.96 cm. This was the highest mean value and was significantly different from all the other treatments. Each of the phytohormones alone had an inhibitory effect on epicotyl growth; jasmonic acid (treatment 3) had the greatest inhibitory effect with a resulting height of 1.16 cm. In treatments 2 (6-benzylaminopurine) and 6 (Ethephon), epicotyl heights reached 1.37 and 1.39 cm, respectively, although there were no significant differences on growth between treatments 3 and 6, 3 and 2, and 2 and 6. A greater inhibitory effect on epicotyl growth was observed with treatment 4 (6-benzylaminopurine: jasmonic acid, 10:10) and 5 (6-benzylaminopurine: jasmonic acid, 10:100) with a resulting mean height of 1.08 and 0.73 cm, respectively. The increase in the concentration of jasmonic acid resulted in a greater inhibitory effect on growth. A similar effect was also observed in treatments 7 (6-benzylaminopurine: Ethephon 10:10) and 8 (6-benzylaminopurine: Ethephon, 10:100): 1.22 to 1.01 cm, though it was less intense. The three phytohormones together, at concentrations of 10 µM each (treatment 9: 6-benzylaminopurine: Ethephon: jasmonic acid, 10:10:10), produced a mean growth of 1.11 cm. Increasing Ethephon concentration from 10 µM to 100 µM in treatment 10 (6-benzylaminopurine: Ethephon: jasmonic acid, 10:10:100) produced a mean epicotyl height of 0.83 cm. The greatest inhibitory effect was associated to treatment 11 (6-benzylaminopurine: Ethephon: jasmonic acid, 10:100:10) in which the mean epicotyl height was 0.54 cm.

Bud induction

The mean number of induced buds under the different treatments is presented in figure 3. The ANOVA

TABLE 1.

Effects of different phytohormone treatments on epicotyl growth: probabilities after post hoc Tukey tests.

Treatment	1	2	3	4	5	6	7	8	9	10	11
1	-	**0.000015**	**0.000015**	**0.000015**	**0.000015**	**0.0000154**	**0.000015**	**0.000015**	**0.000015**	**0.000015**	**0.000015**
2		-	0.747647	**0.013765**	**0.000061**	1.000000	0.770004	0.173802	0.471314	**0.001401**	**0.000015**
3			-	0.999783	0.164479	0.887991	0.999973	0.997178	1.000000	0.524193	**0.003068**
4				-	0.221333	0.232560	0.777952	0.999992	1.000000	0.694268	**0.002134**
5					-	**0.001280**	**0.007288**	0.836005	0.311870	0.999973	0.988737
6						-	0.954028	0.364062	0.713624	**0.013608**	**0.000018**
7							-	0.872461	0.997010	0.079254	**0.000024**
8								-	0.999892	0.988737	0.145358
9									-	0.732346	**0.009613**
10										-	0.836005
11											-

test showed highly significant differences between treatments ($P < 0.0001$). Post hoc analysis with the Tukey test indicated several significant differences between pairs (Table 2). The control treatment (1) was not statistically different from treatments 3 (jasmonic acid) and 6 (Ethephon), in which only the apical buds were developed; the other treatments produced a significantly greater number of buds. Treatment 2 (6-benzylaminopurine) increased significantly the number of buds to a mean 4.78, which represented the highest one obtained in this study. The GLM model for regression analysis yielded a significant P value (<0.0001) and explained a 49% of the variability. The equation obtained was: number of buds = 1.3680 + 0.3046*BA – 0.0747*JA - 0.0211*E + 0.0006JA^2 + 0.0002*E^2, where BA is the effect attributed to 6-benzylaminopurine, JA is that of jasmonic acid and E is that of Ethephon. The single and quadratic effect of Ethephon were not significant, while the effects of 6-benzylaminopurine and jasmonic acid (both single and quadratic) were highly significant. The parameter esti-

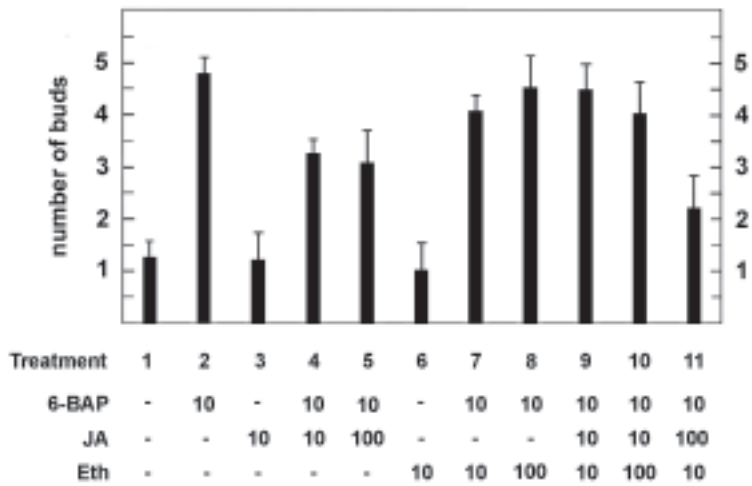

FIGURE 3. Mean values for the number of lateral buds induced in the different treatments. Data are the mean ± standard deviations.

TABLE 2.

Effects of different phytohormone treatments on number of induced buds: probabilities after post hoc Tukey tests.

Treatment	1	2	3	4	5	6	7	8	9	10	11
1	-	**0.000015**	1.000000	**0.000015**	**0.000048**	0.999927	**0.000015**	**0.000015**	**0.000015**	**0.000015**	0.237964
2		-	**0.000015**	**0.000015**	**0.000102**	**0.000015**	0.053511	0.999481	0.994751	**0.522909**	**0.000015**
3			-	**0.000015**	**0.000443**	0.999996	**0.000015**	**0.000015**	**0.000015**	**0.000015**	0.378404
4				-	0.999996	**0.000015**	**0.008991**	**0.016676**	**0.005002**	0.5353718	0.121759
5					-	**0.000082**	0.162856	0.059008	**0.039255**	0.603875	0.700403
6						-	**0.000015**	**0.000015**	**0.000015**	**0.000015**	0.163863
7							-	0.979588	0.977280	1.000000	**0.000023**
8								-	1.000000	0.991038	**0.000033**
9									-	0.992458	**0.000019**
10										-	**0.003239**
11											-

mates indicated that the greater effect was produced by 6-benzylaminopurine (estimate= 0.3046), that stimulated the production of lateral buds. This can be interpreted as the number of buds increased at a rate of 0.3 by unit of 6-benzylaminopurine.

The effect of jasmonic acid alone on the number of buds was inhibitory (estimate= -0.0747): a decrease rate of 0.07 buds per unit of jasmonic acid was expected with the 10 µM concentration, while at the expected decrease in number of buds was lower (r^2=0.0006) at the 100 µM concentration. The phytohormone combinations of treatments 4 (6-benzylaminopurine: jasmonic acid, 10:10) and 5 (6-benzylaminopurine: jasmonic acid, 10:100) produced mean values of 3.22 and 3.06 buds respectively. When increasing jasmonic acid concentration from 10 µM to 100 µM in presence of 6-benzylaminopurine, there was no further significant inhibition increase (Table 2). Treatments 7 (6-benzylaminopurine: Ethephon, 10:10) and 8 (6-benzylaminopurine: Ethephon, 10:100) induced 4.06 and 4.50 buds respectively. As it can be observed in figure 3, treatments 7, 8, 9 and 10 did not present significant differences in the mean bud number (4.06; 4.50; 4.45; 4.00). Those values represented a significant increase in relation to the control treatment (1). Finally, medium 11 (6-benzylaminopurine: jasmonic acid: Ethephon, 10:100:10) with 2.19 buds per explant showed an intermediate level of response between treatments 1, 3 and 6, which produced only one bud; treatments 4 and 5, which produced 3 buds; and media 2, 7, 8, 9 and 10, which produced 4 or more buds. These data confirmed that jasmonic acid had a stronger inhibitory effect than of Ethephon on bud induction. On the other hand, there was a concentration effect, i.e., in presence of 6-benzylaminopurine and Ethephon, inhibition was higher with 100 µM than with 10 µM of jasmonic acid.

Cluster analysis

Main components analysis (SPAD) was performed on the data reported above, with the objective of correlating the global effects of the treatments used, which were grouped according to the recorded height and bud number. A cluster analysis represented in a direct hierarchical tree grouped the 11 treatments in three groups (Fig. 4). In the first group, treatments 2, 7, 8 and 9 were included; in the second one, treatments 4, 5, 10 and 11; and, in the third one, treatments 1, 3 and 6.

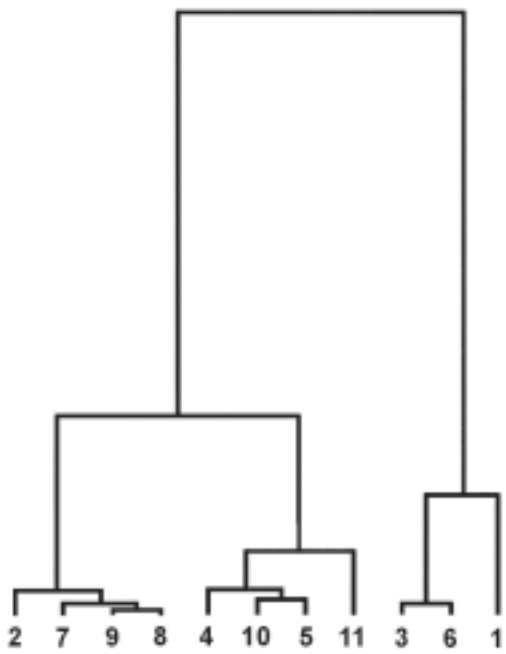

FIGURE 4. SPAD analysis of the main components for height and bud number cluster (represented in direct hierarchical tree) showed the existence of three groups of treatments.

Discussion

Plant responses to exogenous or endogenous phytohormones are usually the result of the activation of more than one signalling and response pathway. In this study, *Pinus pinaster* seedlings with the radicle cut were submitted to 11 different phytohormones combinations. The responses to these conditions were measured in terms of epicotyl growth and the number of lateral buds induced.

Epcotyle growth diminished significantly in relation to the control medium in presence of jasmonic acid, 6-benzylaminopurine or Ethephon, either alone or in combination. The treatments with concentrations of 100 µM jasmonic acid and Ethephon had a stronger inhibitory effect than the treatments with 10 µM. As it can be observed in figure 2, if different treatments that contain one, two or three phytohormones in concentrations of 10 µM (2, 3, 4, 6, 7 y 9) are compared in pairs, the *P* values over 0.05 show there are no significant differences except in case 2:4. In this comparison, the inhibitory effects of jasmonic acid were slightly added to those of 6-benzylaminopurine. Strangely enough, not significant differences were observed when 6-benzylaminopurine was combined with either Ethephon alone or jasmonic

acid plus Ethephon. In other words, each of the phytohormones had on its own, the same inhibitory effect on epicotyl growth as when combined in concentrations of 10 µM. Comparing with one another the media in which at least one of the phytohormones was present at a concentration of 100 µM (5, 8, 10 and 11), a *P* value over 0.05 was observed. This pointed that the growth inhibition produced was not significant and that all these treatments form a more homogenous group than the one with concentrations of 10 µM. The comparison of the media of 10 µM and 100 µM presented extreme cases like medium 8 (8 vs. 2, 8 vs 3, 8 vs. 4, 8 vs. 6, 8 vs. 7 and 8 vs. 9) without significant differences, and the opposite case of medium 11 (11 vs. 2, 11 vs. 3, 11 vs. 4, 11 vs. 6, 11 vs. 7 and 11 vs. 9). The rest of the comparisons gave intermediate results which are also more complex to interpret. In addition to that, and although values did not reach statistical significance, jasmonic acid had a stronger inhibitory effect than Ethephon in the tried combinations. When treatments 3 vs. 6, 4 vs. 7, 5 vs. 8 and 11 vs. 10 were compared, *P* values over 0.05 were obtained which showed that differences were not significant. However, the comparison of 6-benzylaminopurine alone or in combination with jasmonic acid (2 vs. 4 or 2 vs. 5) gave null *P* values which showed that values obtained were different; this was not the case for the combined treatment of 6-benzylaminopurine and Ethephon (2 vs. 7 and 2 vs. 8).

Regarding the number of induced buds, there were no significant differences between the control (treatment 1) and the jasmonic acid and Ethephon treatments. However, jasmonic acid had a strong bud-inducing effect (Fig. 3). The concentrations of 10 µM or 100 µM of jasmonic acid combined with 6-benzylaminopurine (4 and 5) did not produce any significant differences in bud number. The same was observed for the concentrations of 10 µM or 100 µM of Ethephon with 6-benzylaminopurine (7 and 8). This indicated that the difference of concentration did not intervene in the number of induced buds; it also pointed that it was important to consider the type of phytohormone to combine with 6-benzylaminopurine. Jasmonic acid had an inhibitory effect that Ethephon did not produce. The comparison of the p-values of 4:7 and 4:8 showed that the differences were significant. The identical response of treatments 2, 7, 8, 9 and 10 showed that there was no difference between the combinations of one, two or three phytohormones, as long as 6-benzylaminopurine was combined with Ethephon at 10 µM or 100 µM with or without jasmonic acid at 10 µM. In all these media, four or more buds per explant were induced. Treatment 11 with its mean two buds per explant, showed the inhibitory effect of 100 µM of jasmonic acid on bud number and got statistically near to the values of treatments 1, 3, and 6 (one bud), and also of the values of media 4 and 5 with about three buds per explant. Jasmonic acid is usually in the picomolar range in leaf tissue, while other tissues exhibit more than 10 times such level, suggesting that jasmonic acid may act in a variety of physiological mechanisms in non-leaf tissue (Wasternack and Hause, 2002). Our results in *Pinus pinaster* showed that 6-benzylaminopurine induced bud formation, while jasmonic acid had an inhibitory effect; Ethephon, however, showed only an inhibitory effect in medium 11. Moreover, high levels of jasmonic acid in combination with Ethephon strongly inhibited the induction of lateral buds, a situation that was not observed in absence of Ethephon.

The global effect of the different treatments caused on height and bud number was grouped by cluster analysis. Group 1 was composed by treatments 2, 7, 8, and 9, which showed a large mean number of buds (4-5) and a mean growth of 1-1.5 cm. This confirmed the bud-inducing effect of 6-benzylaminopurine and the positive effect of the different phytohormone combinations (6-benzylaminopurine and Ethephon at both concentrations, and the combination of 6-benzylaminopurine, jasmonic acid and Ethephon, but only at 10 µM).

However, group 2 was composed by treatments 4, 5, 10, and 11, which showed 2-4 buds and an epicotyl growth of 0.5-1.2 cm, indicated the inhibitory effect of jasmonic acid on the effect of 6-benzylaminopurine.

Finally, the control treatment (treatment 1) was put together with treatments 3 and 6 in group 3, were the bud number was 1 and growth was only 1.2-2 cm. This showed that buds were only obtained if 6-benzylaminopurine was added to the culture medium.

The complexities of the interactions between the different hormonal treatments will require further studies. However the results presented in the current one indicated that similar signalling pathways act in jasmonic acid and ethylene regulations in a Gymnosperm.

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