

***In Vitro* Multiplication of 'Flordaguard' Rootstock: Cytokinin Source and Concentration Effects, Explants Orientation and Period of Permanence in the Culture Medium**

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ABSTRACT

This work aimed at studying the effects of different sources and cytokinin concentrations, as well as explants orientation and time of their permanence in shoots induction medium to obtain high multiplication rate. Four experiments were carried out to evaluate the sprout percentage mean, shoots number per explant, elongated shoots percentage and shoots length. The treatments with 2iP and zeatin did not promote in vitro plant multiplication. When different BAP concentrations were tested, an increase in sprout percentage was obtained using BAP concentration up to 3.66 mg L⁻¹ (80%), and for shoots number per explant a linear behavior was observed, showing a mean of four shoots per explant with 4.0 mg L⁻¹ BAP. For the 'Flordaguard' rootstocks, the explants orientation did not alter the potential in vitro multiplication and the shoots growth. However, the explants permanence in the shoots induction medium for 40 days provided the best results for shoots growth.

Key words: Micropropagation, cytokinin, explant, *Prunus*

INTRODUCTION

In Brazil, *Prunus* rootstocks are commercially produced through the peaches seeds from preserve industries. However, this method, contrary to the asexually propagation, causes lack of plant standard not allowing the maintenance of the mother plant characteristics in the descendants (Fachinello and Bianchi, 2005).

The clonal propagation is the favorite method in many parts of the world, because it allows the

production of more uniform offsprings. However, this propagation form is difficult in some *Prunus* genotypes due to the low rhizogenesis potential, mainly when the multiplication is made by cutting (Fachinello, 2000).

In these circumstances, the *in vitro* propagation is a viable alternative. Besides increasing the multiplication capacity, it improves the health conditions of the plants produced, resulting the vegetative material in larger amount in a shorter period of time (Silva et al., 2008; Souza et al.,

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2006). However, it is recommended as a technique for the propagation of several annual species and some woody species to obtain high multiplication rates. In the *Prunus* genus some species present many multiplication problems, generating a great variability and sometimes not allowing reproducible results.

Normally, for *in vitro* plant multiplication cytokinin source and concentration are the factors that more influence the apical dominancy overcoming. However, using this plant growth regulator in excess can be dangerous. The plant effects are characterized, mainly, for the lack of explants growth, leaves size reduction, internodes shortening, stems thickening and general hyperhydricity, which conduce to a serious problem in the rooting phase (Santos-Serejo et al., 2006). Several works have been made with *Prunus* species using different BAP concentrations, varying from 0.1 to 6.0 mg L⁻¹, confirming the efficiency of this plant growth regulator, which can change according to species or cultivar (Reeves et al., 1983; Parffit and Amehdi, 1986; Silva et al., 2003; Wagner et al., 2003; Teixeira et al., 2004). Another factor that can also contribute significantly for the success during the multiplication phase is the explants orientation (Yae et al., 1997). However, few studies have been accomplished with the *Prunus* genus, using this kind of treatment.

Nowadays, in Brazil, with the availability of alternative rootstocks to the traditional Aldrichi and Capdeboscq cultivars, there is the possibility to study and to improve the protocols of *in vitro* propagation for the new ones, with the aim of turning the technique economically viable. Among these new rootstocks, the Flordaguard cultivar is a hybrid from *Prunus persica* x *Prunus davidiana*. This rootstock has been mentioned as a promising alternative for Brazilian conditions, because of its show little requirement cold, resistance to the two nematode species (*Meloidogyne javanica* and *M. incognita*) more commonly found in Rio Grande do Sul-Brazil, as well as possible tolerance to the *Mesocriconema xenoplax*, the primary agent related to the Prunus Tree Short Life disease (Carneiro, 1998).

The aim of this work was to study the *in vitro* propagation of 'Flordaguard' rootstock, where it was tested the best source and cytokinin concentration effects, explants orientation and their permanence in the culture medium. It also aimed at evaluating the potential propagation as

well as to establish a viable protocol to produce a large quantity of this rootstock *in vitro*.

MATERIAL AND METHODS

The 'Flordaguard' rootstock was grafted on 'Capdeboscq' cultivar and the plants were maintained in greenhouse in vases (10 L) filled with sterilized soil. The plants received nitrogen fertilization (NO₃) with the purpose to stimulate the development of new auxiliary shoots to use for *in vitro* establishment. When the plants started to sprout, they were sprayed with agrimicina (2.4g L⁻¹) and captan (1g L⁻¹) at each three days to reduce the *in vitro* contamination. During the plenty growth vegetation, micro-shoots with 1 cm and one bud were established *in vitro*. These were inoculated in test tubes containing MS medium (Murashige and Skoog, 1962) with 25% salt reduction, containing myo-inositol (100 mg L⁻¹), sucrose (30 g L⁻¹), agar (6 g L⁻¹) and pH 5.9. The *in vitro* multiplication phase was accomplished in three stages, in a total of four experiments as follows.

Experiment 1: Cytokinin source and its concentration effects in the *in vitro* multiplication of 'Flordaguard' rootstock

In this experiment, the effects of three different cytokinin sources was evaluated: benzylaminopurine (BAP), isopentenyladenine (2iP) and zeatin (ZEA), in different concentrations (0.0; 0.5; 1.0; 1.5 and 2.0 mg L⁻¹). As basic medium, the salt from MS medium was used, with 50% of the nitrogen sources, added by MS medium vitamins, AIB (0.01 mg L⁻¹), myo-inositol (100 mg L⁻¹), sucrose (30 g L⁻¹), agar (6 g L⁻¹) and pH 5.9. Apical shoots with approximately 1.0 cm, from the previous cultured phase were used as initial explants. The culture was conducted in a growth chamber at 25 ± 2 °C, with 16 h of photoperiod and 25 μmol m⁻²s⁻¹ of photosynthetic photon density for 40 days. Later, the percentage of explants sprouted, the number of shoots per explant, and their average length was analyzed.

Experiment 2: BAP concentration increase to improve the multiplication rates of 'Flordaguard' rootstock

This experiment was conducted to study the influence of BAP concentration higher than tested in the first experiment. As initial explants, apical

shoots from the previous multiplications, with approximately 1.0 cm, were used. These were cultured in MS medium with 50% of nitrogen sources added with BAP (0.0; 1.0; 2.0; 3.0 and 4.0 mg L⁻¹). The other medium reagents, as well as the cultivation conditions and the analyzed variables were the same ones of the previous experiment.

Experiment 3 and 4: Explants orientation and the time of permanence in the shoots induction medium to improve the shoots number per explant (experiment 3) and shoots growth (experiment 4)

These two experiments aimed at studying the influence of explants orientation and their period of permanence in the medium to improve the shoots induction (experiment 3) and in a second stage, the effect of those factors for the growth of the new shoots (experiment 4).

Experiment 3: Initially, the explants (apical shoots from the previous multiplications, with approximately 1.0 cm) were planted in MS medium with 50% of nitrogen sources added with BAP (4.0 mg L⁻¹), sucrose (30 g L⁻¹), AIB (0,01 mg L⁻¹), 100 mg L⁻¹ of myo-inositol (100 mg L⁻¹), agar (6 g L⁻¹) and pH 5.9, with the purpose of inducing new shoots. Fifty percent of the explants were placed in the medium in vertical orientation and 45° inclined orientation. After planted in the pots, they were transferred to a growth chamber with the same cultivation conditions as the first experiment.

Experiment 4: Twenty, 30 and 40 days after the trial setting, the explants of both the positions were transferred to medium with the same basic composition, but with BAP concentration reduction (0.5 mg L⁻¹), with the purpose of evaluating the shoots growth.

The evaluations were performed in two stages; the first one was done when the explants were transferred to the medium with BAP (0.5 mg L⁻¹), and the percentage and number of shoots per explants were evaluated (experiment 3). After 40 days in the medium with 0.5 mg L⁻¹ of BAP, the percentage of shoot growth and the explants average length were evaluated (experiment 4).

The apical shoots, with approximately 1.0 cm, used as initial explants in the second and third experiment came from plantlets cultivated in macro and micronutrients from MS medium with BAP (2.0 mg L⁻¹). After four to five successive cultures in this medium, the explants were

transferred to the same basic medium, but without BAP, and then used as explants in the experiments.

Experimental design and statistical analysis

All the experiments were carried out in a completely randomized design, with five repetitions. Each experimental unit was composed of a flask containing four explants. The results were submitted to the variance analysis, and the cytokinin source, explants orientation and time of permanence in the induction medium factors were submitted to multiple comparison using Duncan Test. The regression test for the BAP concentration factor was carried, at 5% of significance level, using SANEST as statistical software (Zonta and Machado, 1984).

RESULTS AND DISCUSSION

Cytokinin source and cytokinin concentration effects

There were no significant effects on the explants multiplication with different 2iP and zeatin concentration as treatments. Therefore, the BAP levels were analyzed just by the regression. For the explants sprouting percentage (Fig. 1A) and number of shoots per explants (Fig. 1B), which showed a linear response to different BAP concentrations. However, during the 40 days that the explants were kept in the medium, there was no treatment effect on the shoot length variable.

Results showed for 'Flordaguard' rootstock *in vitro* multiplication, BAP addition was necessary when MS culture medium was used, because in this culture stage, the break apical dominance was necessary to induct the axial sprout proliferation, this effect was promoted by cytokinin. The *in vitro* multiplication rates were also affected by the cytokinin source and concentration used in the culture medium, which was in agreement with Grattapaglia and Machado (1998).

Results also showed of BAP superiority in relation to the 2iP and the zeatin was observed which in agreement with most of the *in vitro* propagation studies, where BAP was effective to promote *in vitro* multiplication and seemed to be the best cytokinin for adventitious bud induction and aerial parts multiplication. Moreover, among all, BAP is the most economical cytokinin (Augusto, 2001; Silveira et al., 2009).

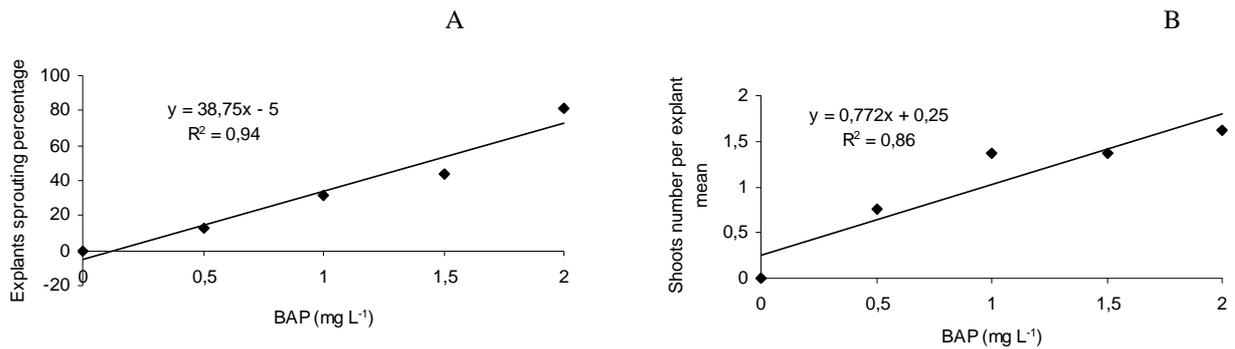


Figure 1 – Explants sprouting percentage (A) and shoots number per explant mean (B) obtained with the 'Flordaguard' rootstock cultured in MS medium with different BAP concentrations.

Studying *in vitro* multiplication behavior of 'Hansen 2' and 'Hansen 5' peach rootstocks, Martinelli (1985) observed a larger shoot number with 0.6 to 1.0 mg L⁻¹ BAP, obtaining an average of three new shoots per explant, while 2iP and kinetin just promoted the explants sprouting. In an experiment with pear, Shibli et al. (1997) observed a larger *in vitro* multiplication rate when the explants were cultured in medium containing BAP, when compared to zeatin, obtaining 7.6 and 2.8 of new shoots per explant, respectively, with 1.0 mg L⁻¹ of both plant growth regulators. Similar result was obtained by Augusto (2001) comparing the effect of 1.0 and 2.0 mg L⁻¹ of different cytokinin sources, during the blueberry cv. Brazos *in vitro* multiplication; who observed that 2iP, zeatin, kinetin and TDZ produced fewer number of new shoots per explant in relation to BAP. In this experiment an average of 1.5 and 4.3 shoots per explant, were obtained using 2iP and BAP, respectively.

The lower *in vitro* multiplication rate obtained with 2iP and zeatin was due to the that these were natural cytokinins, and consequently they were degraded by the cytokinin oxidize enzyme action, which broaks the lateral chain of these molecules. However, the larger degree of 2iP degradation (Barrueto Cid, 2000) could explain the poorer results obtained using this cytokinin source.

There was no *in vitro* multiplication and elongation from the explants cultivated in the medium with 2iP and zeatin, but leaves grew and development from the initial explants was observed. Besides these characteristics, rooting in some explants cultivated in the medium with 2iP was observed, which was related to a larger action of cytokinin oxidize upon 2iP in relation to the

zeatin (Peres and Kerbauy, 2004), possibly altering the hormone balance, driving to a higher auxin/cytokinin relation and explants rooting.

Several studies have reported better action from other cytokinins, these were in agreement with the studies carried out with highbush blueberry, where zeatin and 2iP were used commonly for *in vitro* multiplication (Eccher and Noé, 1989; Popowich and Filipenya, 1997; Gonzales et al., 2000; Debnath, 2004). However, the zeatin induces a larger shoot number per explant in comparison with the 2iP. On the other hand, no significant differences were observed by Ribas et al. (2005), using zeatin and BAP for *in vitro* multiplication of *Aspidosperma polyneuron*, but they had better results with 2iP in relation to the kinetin. These studies supported that species or cultivars from the same species can have different *in vitro* multiplication behavior, caused by different cytokinin sources. They supported Grattapaglia and Machado (1998) results, that the growth and the development pattern of most *in vitro* cultivations were related directly with medium drugs composition and plant growth regulators concentrations used in the culture medium. Nevertheless, the suitable amount of plant growth regulators can change according to their endogenous concentration and the kind of plant tissue.

Due to the low *in vitro* multiplication rate obtained in the first experiment, a second one was carried out with the objective of verifying the effect of BAP concentration increase in the *in vitro* multiplication rate of 'Flordaguard' rootstock. Regarding the behavior obtained with 2iP and zeatin in the first experiment, the cytokinin sources were not tested again, because, besides

their high cost, probably, the exogenous addition of those cytokinins should be higher in comparison with BAP to obtain the positive results for *in vitro* plant multiplication.

Effect of BAP concentration increase

The BAP concentration increase in the culture medium provided a significant effect on the explants sprout percentage and shoot number per explant. Based on the regression analysis, an estimate increase on explants sprout percentage were observed until 3.66 mg L^{-1} BAP

concentration in the culture medium (Figure 2A). However, with the BAP concentration increase, a linear behavior was observed with an increase of shoot number per explant (Figure 2B).

In comparison to the first experiment, in this experiment the shoots number increased with the BAP concentration increment. However, it was not followed by the shoots growth. At the highest BAP concentration, the shoots became bulged, with leaves size reduction, but without hyperhydricity symptoms (Figure 3).

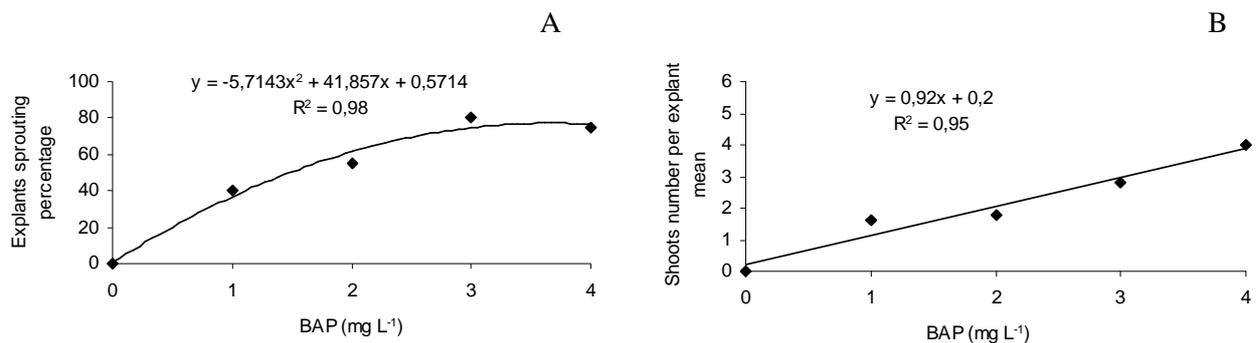


Figure 2 – Explants sprouting percentage (A) and shoots number per explant mean (B) from 'Flordaguard' peach rootstock, cultured in medium added with different BAP concentrations.



Figure 3 - Shoots aspect of 'Flordaguard' rootstock obtained in culture medium with 3.0 mg L^{-1} (A) and 4.0 mg L^{-1} BAP (B).

The useful BAP effects on the shoots multiplication is linked with the influence that this plant growth regulator has on the cellular division and shoots growth induction to the axillaries buds inhibited by the plant apical dominance. However,

the best BAP concentration for the explants multiplication is genotype dependent.

In this experiment, the largest shoots number per explant was observed with 4.0 mg L^{-1} BAP, which was similar the results of Parffit and Amehdi

(1986). They obtained better results with higher BAP concentrations, reaching in some *Prunus* genotypes an average of 9.9 shoots per explant, using culture medium added with 6.0 mg L⁻¹ BAP and 0.01 mg L⁻¹ IBA. On the other hand, Teixeira et al. (2004) did not obtain success in the 'Carelli' *in vitro* multiplication, with BAP concentration increased from 0.5 to 4.0 mg L⁻¹, obtaining an average of 3.4 shoots per explant. This was in agreement with Silva et al. (2003) experiment with GF 677; who obtained an average of 10.5 new shoots per explant in the culture medium added with only 0.5 mg L⁻¹ BAP. Rocha et al. (2009) obtained similar results, where the shoots number increase until the addition of 0,8 mg L⁻¹, with 3.0 shoots per explant. High cytokinin concentrations (3.0 and 5.0 mg L⁻¹) have been used for shoots proliferation in other woody species, with satisfactory results, as well as observed in some forest species (Arellano and Pinto, 1993; Lamb et al., 2004).

The main objective of *in vitro* multiplication phase is to produce the largest number of plants in a short period of time. Apart from the plant growth regulators and their concentration, to obtain good results in this phase it is necessary that the culture medium supplies the essential substances for the *in vitro* plants growth. Gaspar et al. (1996) reported

that when the nutritious medium composition was in agreement with the plant requirement, the multiplication key factor was the presence and concentration of the plant growth regulators, particularly the cytokinins, because *in vitro* multiplication rate was largely controlled by the genotype and cytokinin concentration interaction. According to the results, the 'Flordaguard' rootstock has potential for *in vitro* propagation in the culture medium added with BAP, therefore, the explants need to go through a growth phase, once the shoot quality from the multiplication phase can be decisive for the rooting shoots success.

Explants orientation and the time of permanence in shoots induction medium: effects on proliferation and shoots growth

According to the variation analysis for the shoots percentage, a significant interaction at the studied factors was not observed for any factor. However, the shoots number per explant was influenced by the time of permanence in the culture medium factor, i.e., those explants that stayed for a longer period of time in the shoots induction medium produced a larger shoots number, however, without significant difference between 30 and 40 days, with 4.23 and 4.13 shoots per explant, respectively (Figure 4).

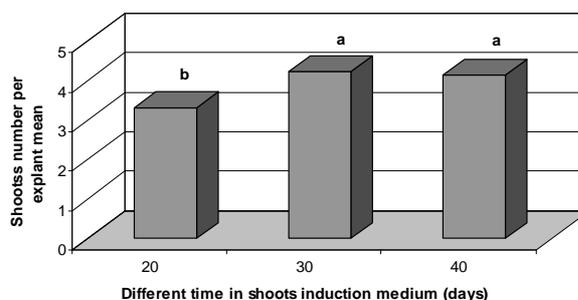


Figure 4 – Shoots number per explant mean from 'Flordaguard' rootstocks, obtained with different times in shoots induction medium.

These results was agreement with most of the works performed, where it has been reported that the most common period between the explants subculture, was around four weeks. On the other hand, species such as *Citrus sunki* (Hort. Former Tan.) need five to six weeks between explants subculture for a larger multiplication rate, because this species present a long adaptation period after the explants change to a new medium, where the

explants multiplication starts just in two or three weeks. However, for the species that has a fast initiation of active growth, subcultures at every three weeks are more suitable (Grattapaglia and Machado, 1998).

Although significant difference between the explants orientation was not observed, a superiority tendency was observed for the explants cultured 45° inclined in relation to vertical

orientation, with 4.06 and 3.72 shoots per explant, respectively.

It is known that source and cytokinin concentration in the culture medium are the main factors influencing *in vitro* plant multiplication. Besides the cytokinin use, with the objective of increasing the shoots number, the explants can receive different treatments, for instance, apex pruning and explants orientation change (horizontal, inclined and inverted). These treatments can influence the axillary shoots proliferation due to reduction in the apical shoots dominance, because, according to Taiz and Zeiger (2004) the auxin transport is polar, and possibly this behavior is important to control the axillary buds development inhibition. In such case, the treatments that reduce or inhibit the explants auxin movement, increase the shoots number per explant.

Several authors have reported the positive effect of this kind of treatment. However, depending on the species as well as genotype, the explants cultured in different orientations, did not show positive

behavior when compared to the explants cultured in vertical position. Similar effect was observed by Chaves (2003), who studying different explants orientation in *Prunus* spp., obtained a larger shoots number per explant in horizontal orientation from the Mr. S. 1/8 cultivar, but no significant differences were observed in the Okinawa cultivar. Similar results were found in a study carried out with apple explants, in which differences were observed among the explants cultivated in horizontal and vertical orientation and in relation to the genotypes tested (Zimmerman and Fordham, 1989; Yae et al., 1997).

When the explants and their respective shoots were transferred to a medium with a reduced cytokinin concentration (0.5 mg L^{-1} BAP), it was observed that the elongated shoots percentage was significantly influenced by the time of permanence in the shoots induction medium. Figure 5, showed that the highest elongated shoots percentage (74%) was obtained when the explants remained in the shoots induction medium for 40 days.

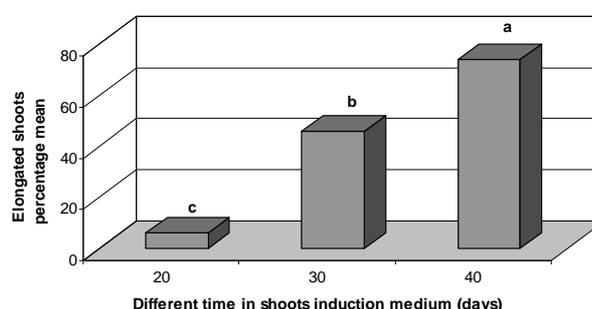


Figure 5 – Elongated shoots percentage mean from 'Flordaguard' rootstock cultured in different times in shoots induction medium.

The results obtained in this study were in agreement with Grattapaglia and Machado (1998), according to them the explants transference for a basic culture medium, diluted or not, and with reduced concentration or cytokinin absence, were enough to promote the shoots growth, before shoots excising for the rooting phase. A significative interaction between the time of permanence in the shoots induction medium and explants orientation was observed for the shoots length mean variable.

The explants cultured in the vertical orientation

grew more in relation to the inclined ones, when they were cultured in the shoots induction medium for 20 and 40 days (Figure 6). For the explants cultured inclined for 40 days, a higher explants length was obtained, but without significant difference in relation to the ones cultured for 30 days in shoots induction medium. Therefore, in a general way, the highest shoots length was obtained when the explants were cultured in vertical position for 40 days in the shoots induction medium, with a mean of 1.54 cm (Figure 6).

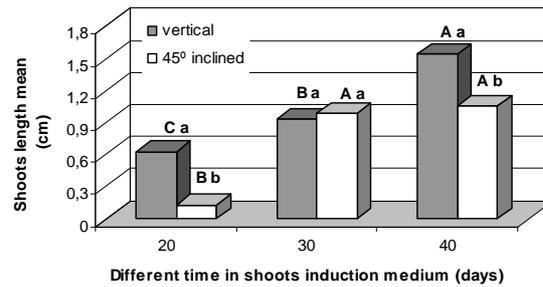


Figure 6 – Shoots length mean of 'Flordaguard' rootstock cultured in vertical and inclined orientation, for different times in shoots induction medium.

Different capital letters differ amongst themselves for time of permanence in the induction medium factor in relation to the explants orientation factor. Different lower-case letters differ amongst themselves for the explants orientation factor in relation to the time of permanence in shoots induction medium, when comparing with Duncan Test ($P > 5\%$).

These results were in agreement with Chaves (2003) and Chevreau and Lebblay (1993), who obtained higher shoots height from Mr. S. 1/8 and Okinawa peach rootstocks and Passe Crassane pear cultivar, respectively, when the explants were cultured in vertical orientation. Contrary to this, McClelland and Smith (1990) obtained the shoots with similar height from McIntosh apple, when the explants were cultured in horizontal and vertical

orientation. On the other hand, for another *Malus* cultivar, like Delicious and Mutsu, the explants cultured with inverted orientation produced the shoots higher in relation to the explants cultured in horizontal and vertical orientation (Zimmerman and Fordham, 1989).

For shoots growth percentage as well as shoots height mean, the best results were obtained when the explants were cultured for 40 days in shoots induction medium, independently from the explants orientation (Figure 5, 6 and 7). In contrast, the explants that were cultured in the shoots induction medium for only 20 days, presented higher number of necrotic shoots, and when cultivated in the medium with reduced BAP concentration, showed only 6% of elongated shoots.



Figure 7 – Aspect of 'Flordaguard' rootstock explants from shoots induction medium, cultured in vertical orientation for 40 days (0.5 mg L^{-1} BAP) (A), and shoots taken from the main explant (B).

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