In vitro morphogenesis from leaves fragments of pear and apple rootstocks

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ABSTRACT

The plant tissue culture represents advantages such as the large scale increase in number of individuals, the possibility of maintaining biodiversity from germplasm conservation. The objective was to verify the *in vitro* morphogenesis of leaves, of the two rootstocks used in apple and pear growing, 'G 874' and 'OHxF 87', respectively. The study is based on the need of obtaining quality materials from *in vitro* propagation technique, which enable a greater homogeneity of the plants, in a short period of time and in addition, a possibility of germplasm conservation. For two different cultivars of rootstocks, experiments were performed, a triple factorial with three types of growth regulators - BAP, 2iP and NAA -, five concentration - 0, 4, 8, 12 and 16 mg L^{-1} - and two leaf cut direction - longitudinally and transversely. Based on the results, it was concluded that the presence of part of the midrib favors the formation of calluses *in vitro* and for callus induction of cultivars OHxF 87 and G 874 is necessary the presence of 4 mg L^{-1} and 8 mg L^{-1} of NAA, respectively. The addition of auxin to the culture medium favors *in vitro* morphogenesis of leaves, as well as the number of calluses, number of roots and root length of 'OHxF 87' and 'G 874'.

Index terms: *Pyrus* sp.; *Mallus* sp.; micropropagation; growth regulators; calogenesis.

INTRODUCTION

For the apple tree crop, of the newly developed rootstocks in the world, the American series Geneva are the most complete in terms of agronomic characteristics required for use in Brazil (Denardi et al., 2015), where stand out the resistance to the wooly aphid, improved branching and angle of the branches, increased yield and tolerance to replanting disease (Fazio; Robinson, 2008). In studies with 'Imperial Gala' and 'Mishima Fuji' apple trees, Pasa et al. (2016) evaluated several rootstocks, including the Geneva series and among the results obtained, the 'G 874' is among those that provide higher yield and in addition, reduce the cross-sectional area of the trunk of both cultivars, but without reducing productivity.

When it comes to the pear tree culture, it is not different, the main problems that block the expansion of this culture in Brazil are related to the vigor of the plants and the type of rootstock used (Fachinello et al., 2011).

The rootstocks of *Pyrus communis* L. are compatible with all European pear cultivars and through plant breeding they may have important characteristics selected such as vigor control, disease resistance, adaptability and precocity (Sharma et al., 2010). Along with this, Brooks's (1984) successful breeding of pear rootstocks was performed from the varieties "Old Home" x "Farmingdale" (OH × F) in Oregon, United States (Robbani et al., 2006), where according to Azarenko et al. (2002), Ercisli et al. (2006) and Mielke (2008), these clones (OHxF) are a good alternative since they induce precocity, control of plant vigor, more uniform and higher quality production and mainly, tolerance to low temperatures and to diseases as decline of pear and fireblight.

Considering the importance of studing these fruit species, the vegetative propagation deserves to be highlighted and mainly, the plant tissue culture represents advantages such as the large scale increase in number of individuals, the possibility of maintaining biodiversity from germplasm conservation (Sartor et al., 2012; Kumar et al., 2016) and production of clonal plants, but also as a basis for the introduction of genetic variation by genetic transformation or mutagenesis (Pérez-Tornero et al., 2010).

Among the *in vitro* establishment techniques of plant tissues, calogenesis is a technique that stands out and consists of using callus culture obtained *in vitro* for subsequent cell arrangement (Zanotti et al. 2012) and is associated with bud and/or roots regeneration from explants without pre-existing meristem, such as leaves, petioles, hypocotyls, protoplasts, among others (Vujovic et al. 2014). Calluses can be multiplied by successive subcultures, maintained *in vitro* for long periods and are of great importance for morphogenetic studies

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and by suspending cells to obtain secondary products, representing a biotechnology of great scientific and commercial interest (Rodrigues; Almeida, 2010), including possible future work on genetic transformation (Erig; Schuch, 2005).

Among the many factors that may affect calogenesis, the age and type of explants, culture conditions, culture medium and growth regulators can be cited, the latter being both qualitative and quantitative, greatly influencing the *in vitro* culture and regeneration of different species (Rodrigues et al., 2009; Stachevski et al., 2013), therefore being necessary to determine efficient protocols for *in vitro* callus induction (Flores; Nicoloso, 2007).

Based on the described problematic, the objective of this study was to determine the influence of different growth regulators, as well as their concentrations, and leaf cutting direction of two different rootstocks used in fruit growing of pear and apple - cultivars OHxF 87 and G 874 - for callus induction from explants already established *in vitro*.

MATERIAL AND METHODS

The study was conducted at the Plant Micropropagation Laboratory of the College of Agriculture and Veterinary of the Santa Catarina State University (CAV-UDESC - Lages/SC).

Two different plant materials were used for the experimental design, rootstocks 'OHxF 87' and 'G 874', being these important for pear tree and apple tree crops, respectively. The explants used for the study were originated from *in vitro* culture, maintained with MS medium (Murashige; Skoog, 1962), with a complete concentration of salts, plus 0.1 g L^{-1} of myo-inositol, 30 g L^{-1} of sucrose and 4.5 g L^{-1} of agar, presented around 5 ± 1 cm in length, after 45 days of the last subculture.

For each cultivar evaluated three different growth regulator were tested: BAP (6-benzylaminopurine), 2iP (2-isopentenyladenine) and NAA (α-naphthalene acetic acid), five concentration (0, 4, 8, 12 and 16 mg L^{-1}) and two levels for the leaf cutting direction - longitudinally (parallel to the leaf's midrib) and transversely (perpendicular to the leaf's midrib). The experimental design was completely randomized, constituting a triple factorial (3 x 5 x 2), Thus, the study comprised 30 treatments for each cultivar, with six replicates of five leaves each, with approximately 1 ± 0.5 cm^2.

The explants were inoculated with the adaxial surface in contact with the MS culture medium. The pH of the culture medium was adjusted to 5.8 ± 0.1 before addition of the agar, which was then autoclaved for 20 minutes at 121 °C under a pressure of 1.5 atm. After inoculation, the explants were kept in the dark for a period of ten days in a growth room at 25 ± 2 °C and after that period, they were transferred to a 16 hour photoperiod, temperature of 25 ± 2 °C and light intensity of 42 µmol m^{-2} s^{-1}, supplied by white fluorescent lamps.

The variables analyzed were: explants oxidation index (%), callus intensity by the scale: 1 - absent, 2 - little (<1 cm), 3 - medium (1-1,5 cm) and 4 - high > 1.5 cm), number of roots, number of calluses with roots and average length of roots (cm). In order to understand the obtained data, was performed the analysis of variance by applying the F test and, when this was significant, was performed the paired comparison of the means by Tukey's test with an error probability of 5% for the qualitative factors and for the quantitative factors , a polynomial regression analysis was performed. The discrete variables, originated from counting, were transformed by the expression [V (x + 0.5)], where x is the mean obtained from each variable and the scale referring to callus intensity in log (x + K) where x is the mean obtained from each variable and K is equal to 1, by the statistical program "R".

RESULTS AND DISCUSSION

Table 1 shows the analysis of variance of the different factors studied in the present article.

The oxidation rate of the explants, as well as the callus intensity, presented different behaviors for the two distinct cultivars, for 'OHxF 87' there was a double interaction between the factors growth regulator and concentration (Figure 1).

As shown in figure 1, there was a quadratic regression in relation to the growth regulators used and, consequently, the concentration of each of them. The use of NAA, when compared with the other regulators,

resulted in lower oxidation of the explants, with 3.15% and 3.20%, respectively, in the concentrations of 12 and 16 mg L^{-1}, respectively, differently in relation to the two cytokinins (2iP and BAP) used, where they characterize maximum values between 68.75% and 84.37% of oxidation between the concentrations of 8 to 12 mg L^{-1}. These results contradict those found by Rabelo et al. (2007), who found that during the induction of calogenesis of *Schizolobium parahyba* explants, the absence of growth regulators caused a higher percentage of oxidation, unlike the morphogenesis studied in this article which, in general, as the concentration of the growth regulator was increased, the oxidation rate was higher. In studies with *Prunus, Pyrus* and *Rubus*, Vujovic et al. (2014) also obtained lower results for callus formation in culture medium with BAP, or even this cytokinin combined with low concentrations of AIB, NAA and 2,4-D and the authors note that the leaf explants of 'Pyrodwarf' pear tree rootstock, after 45 days cultivated with BAP alone, without cytokinin, did not form callus.

Corroborating the results of oxidation indexes, the callus intensity scale was significantly higher with the use of the auxin NAA, at concentrations of 8 and 12 mg L^{-1}, and there were no statistical differences between treatments with the presence of 2iP and BAP, where low levels of calogenesis occur in the *in vitro* leaves of the rootstock OHxF 87. Similarly, Flores and Nicoloso (2007) observed an increase in callus area as the NAA concentration increased in the culture medium and that BAP concentrations, as well as the interaction between BAP and NAA, did not affect the callus area in explants of *Pfaffia tuberosa*. According to Werner et al. (2007), in culture media without growth regulator it is possible to occur inhibition in the callus formation of explants, in

Table 1 – Analysis of variance of the variables: oxidation index (O.I.), callus intensity (C.I.), number of roots (N.R.), number of callus with roots (N.C.R.) and average length of roots (A.L.R.) in relation to the different factors studied.

Variation factor	DF	Medium square				
		O.I.	C.I.	N.R.	N.C.R.	A.L.R.
				'G 874'		
Growth regulator (GR)	2	0.06 ns	2.84 *	10.95*	1.28 *	2.80 *
Cutting the leaf (CF)	1	0.20 ns	0.01 ns	0.33 ns	5.55 *	1.55 *
Concentration regulator (CR)	4	0.27 ns	0.22 *	3.06 *	0.26 *	0.14 *
GR x CF	2	0.001 ns	0.01 ns	0.33 ns	0.31 *	0.04 *
CR x CF	4	0.12 ns	0.01 ns	0.51 ns	0.07 *	0.01 *
GR x CR	8	0.16 ns	0.22 *	3.06 *	0.08 *	0.11 *
GR x CF x CR	8	1.09 ns	0.01 *	0.51 *	0.03 *	0.006 *
Residue	60					
Overall average	-	1.38	1.14	1.98	1.54	1.70
				'OHxF 87'		
Growth regulator (GR)	2	7.77 *	1.93 *	3.01 *	0.19 *	0.40 *
Cutting the leaf (CF)	1	0.01 ns	0.01 *	1.34 *	0.05 ns	0.36 *
Concentration regulator (CR)	4	0.27 ns	0.12 *	2.68 *	0.22 *	0.51 *
GR x CF	2	0.03 ns	0.01 *	1.37 *	0.02 ns	0.10 ns
CR x CF	4	0.06 ns	0.005 ns	1.19 *	0.02 ns	0.15 ns
GR x CR	8	0.67 *	0.13 *	2.70 *	0.25 *	0.62 *
GR x CF x CR	8	0.09 ns	0.005 ns	1.19 *	0.03 *	0.21 *
Residue	94					
Overall average	-	1.57	1.13	1.15	1.75	1.79

* Significant by the Tukey test at 5% probability of error; ns Not significant by the Tukey test at 5% probability of error.

this study, morphogenesis was promoted in the control (without addition of cytokinin or auxin), however, the assigned scale was much lower when compared to treatments with NAA presence. In the same sense, Rabelo et al. (2007) and Aragão et al. (2011) also concluded in studies with woody plants, that in the treatments with MS culture medium, without growth regulator, the explants used did not undergo calogenic inductions or had low callus percentages, respectively.

Differently then this study, Tang et al. (2008), in experiments with four commercial *Pyrus* species (*Pyrus communis, P. pyrifolia, P. bretschneideri* and *P. ussuriensis*), concluded that the cutting method influences the intensity of callus formation in the explants, where regeneration occurred more frequently in the basal section of the leaf, than in the central one, whereas in the apical part no calogenesis occurred.

In contrast, when studying 'G 874', it was found that oxidation was not influenced by any of the treatments and thus, there were no statistically significant differences between them (data not shown). This result, with low oxidation rates regardless of the treatment studied is very interresting, since according to Aragão et al. (2011), phenolic oxidations are one of the main causes of failure of *in vitro* vegetative propagation of woody plants, as in this study case, in which are worked with two fruit species.

The occurrence of calogenesis for the 'G 874' rootstock was determined by the interaction of the three factors evaluated (Figure 2).

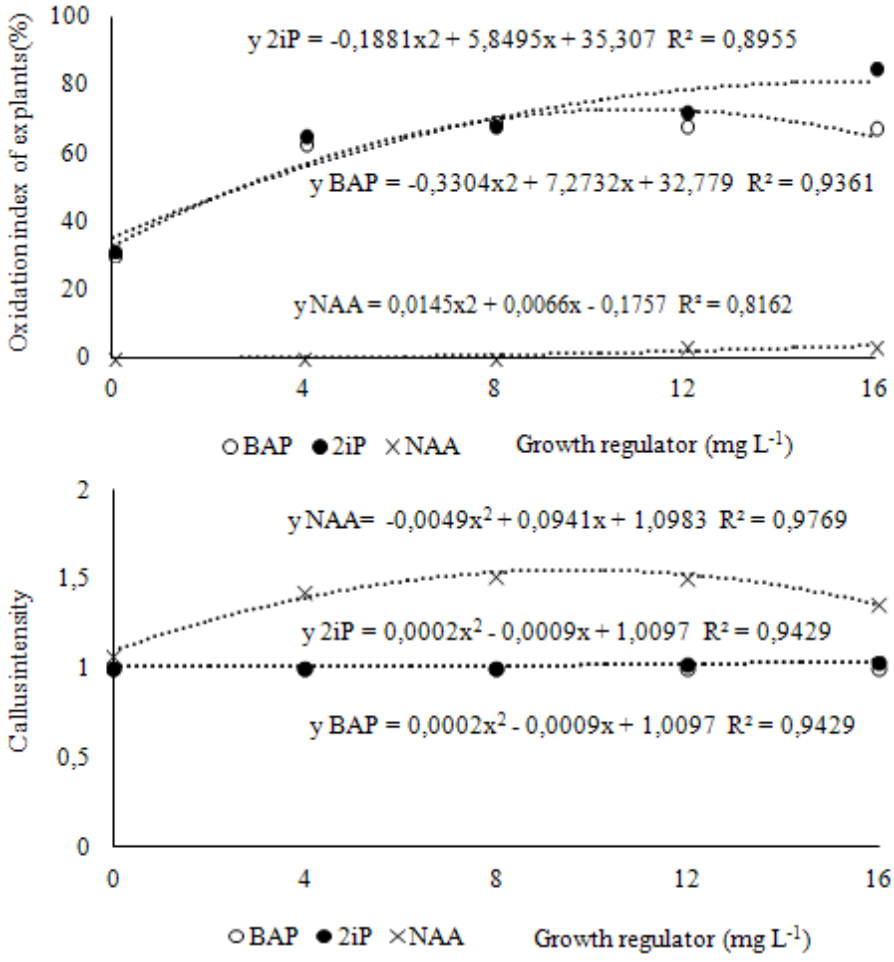

Figure 1 – Percentage of explants oxidation (%) and callus intensity scale in relation to different growth regulators studied and their concentrations, during *in vitro* morphogenesis of 'OHxF 87' leaves.

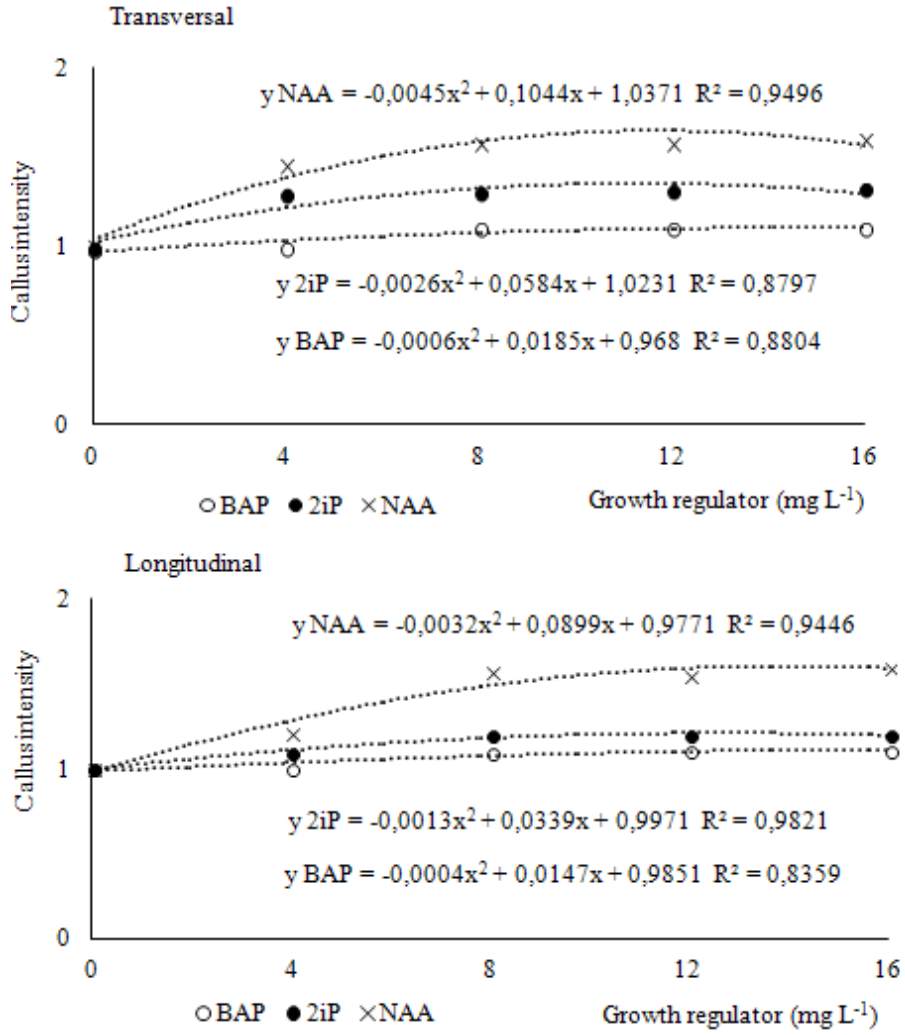

Figure 2 – Scale of callus intensity in relation to different growth regulators studied, their concentrations and leaf cutting direction, during the *in vitro* morphogenesis of 'G 874' explants.

Distinctly from cv. OHxF 87, the apple tree rootstock G 874 presented a triple interaction effect for the callus intensity variable, as shown in Figure 2. For both longitudinal and transverse leaf cuts, the use of NAA promoted a higher formation of calluses in the explants, with emphasis on auxin concentrations of 8, 12 and 16 mg L^{-1}. This proves that the exogenous supply of growth regulators to the culture medium is in many cases necessary for calogenesis induction (Pêgo et al., 2013; Rosa; Dornelas, 2012).

It should be pointed out that in treatments studied for 'G 874' explants, with the presence of the cytokinin 2iP, there was a higher calogenic rate when the cut direction studied was transversely, where part of the midrib was maintained in the explant, which favored the growth of calluses throughout the leaf area. In addition, Erig & Schuch (2005), when studying the morphogenesis of apple tree leaves, also verified that the highest percentages of regeneration and intensity of callus formation were obtained with the transverse thin leaf fragment (transverse) compared to the longitudinal one.

Along with this, Tang et al. (2002) observed that *in vitro* cherry shoots arose from the leaf midrib or in association with vascular tissue, so it is justified to study the influence of explant type on callus induction, where it is recommended the use of those that contain a greater

proportion of meristematic tissue, or that have a greater capacity to express totipotency (Grattapaglia and Machado, 1990). Moura et al. (2008) also point out that in order to induce callus, any plant tissue can be used as an explant, however, with greater expression for morphogenesis, especially young tissues, as in the case of this study, where it was used as source of explants, juvenile material, already established and cultured *in vitro*. Kumar et al. (2016) also emphasize the importance of the explant type study in the *in vitro* callus induction of 'Golden Delicious', where they verified that the highest callus frequency occurred when the leaves were horizontally sectioned and inoculated in the culture media abaxially , as well as, Mitic et al. (2012), in 'Golden Delicious' and 'Melrose' where they obtained differences when using abaxial or adaxial explants.

In Figure 3 is presented the polynomial regression with quadratic behavior for the variable number of calluses with root presence for rootstock 'OHxF 87'. As a consequence of the higher intensity of calogenesis already discussed, the presence of the auxin NAA at the highest concentration (16 mg L^{-1}) favored the development of the root system of the explants, resulting in a greater number of roots, with an average of 2.57 and 2.14 for transverse and longitudinal cuttings, respectively. As in the present study, studies prove that the addition of excessive amounts of auxin to the medium stimulates callus production, although calogenesis can occur even at low concentrations (Grattapaglia; Machado, 1990).

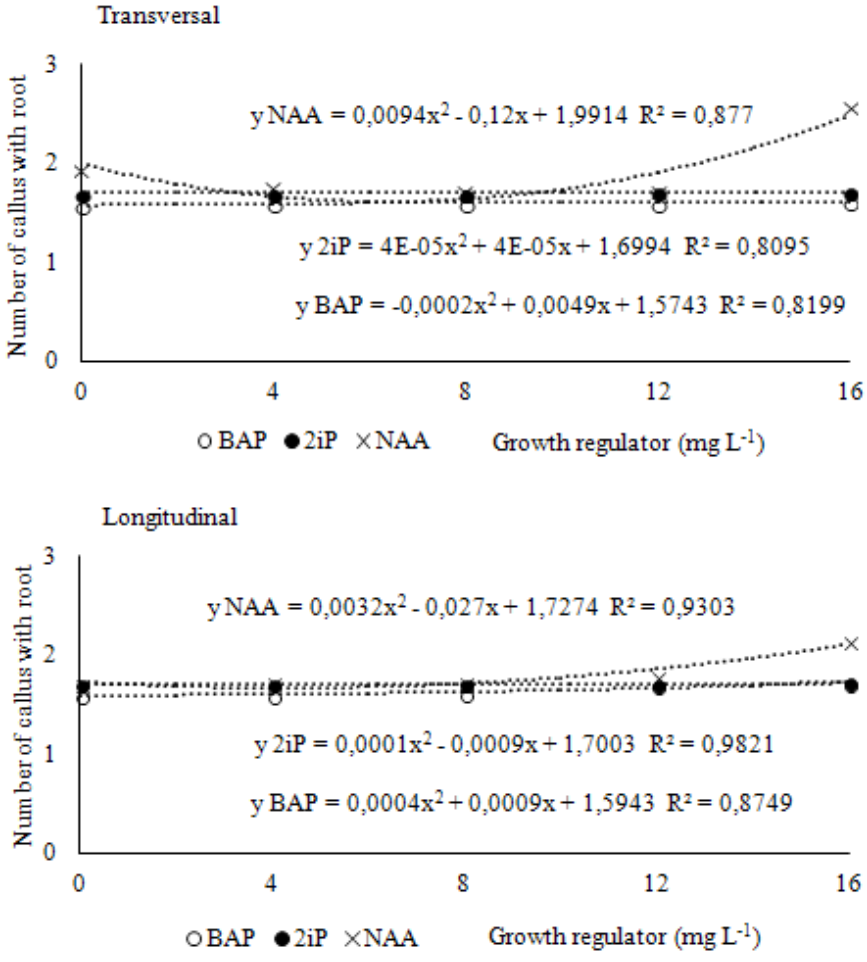

Figure 3 – Number of calluses with roots in relation to different growth regulators studied, their concentrations and leaf cutting direction, during the *in vitro* morphogenesis of 'OHxF 87' explants.

Vujovic et al. (2014) also studying the regeneration of leaf explants from pear tree rootstock 'Pyrodwarf 'concluded that the culture medium with cytokinin alone did not promote root formation in the formed calluses, whereas in presence of auxin, the result was significantly higher. As verified for the cultivar OHxF 87, the presence of BAP alone did not inhibit root formation in the developed calluses, as well as 2iP, however, were statistically inferior to the treatments with different concentrations of NAA in the MS culture medium.

In the same way, for cv. G 874, the presence of auxin provided a higher number of calluses with roots (Figure 4) for both cutting directions, however, in the treatment with 16 mg L^{-1} of NAA in contact with the explant presenting the midrib (tranversally), the number of calluses was 2.2, while in the longitudinal cut, a lower mean of 1.83 calluses with root formation. It is verified with this data that the highest concentration of auxin tested was favorable for the root formation in callus, differently from that found by Flores & Nicoloso (2007), where it was verified that the root formation was negatively influenced by the increase not only of NAA, but also of BAP. The same authors still report that the use of this cytokinin may inhibit the regeneration of axillary shoots and roots, which was not verified at the present, however, compared to the auxin tested, the root system formation was inferior and with significant difference between the treatments.

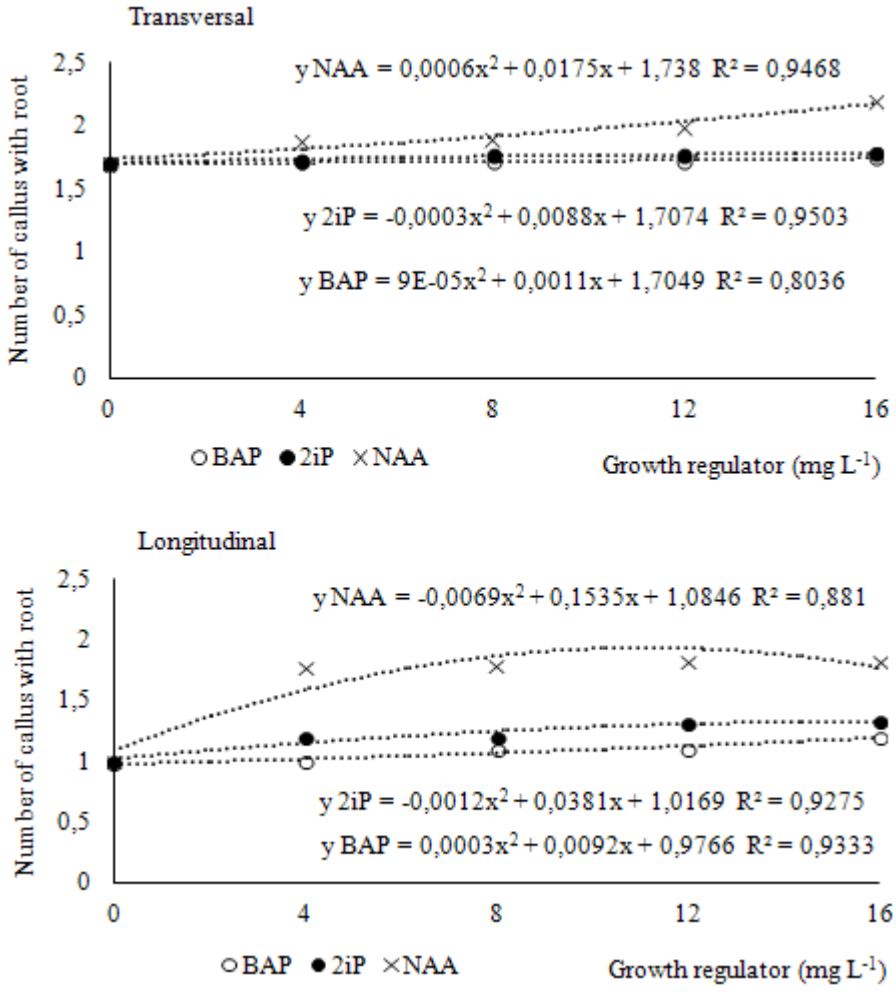

Figure 4 – Number of calluses with roots in relation to different growth regulators studied, their concentrations and leaf cutting directions, during the *in vitro* morphogenesis of 'G 874' explants.

In studies with apple tree, cultivar Golden Delicious, Kumar et al. (2016) found that the percentage of *in vitro* calluses with roots significantly varied according to the concentration of the auxin tested in the culture medium (IBA - indole-3-butyric acid), with the best rooting (96.66%) obtained with IBA at 1.0 mg L^{-1}, a concentration much lower than that found in this study, where for 'G 874', the highest occurrence of calluses with root presence was with 16 mg L^{-1} of the auxin NAA.

When considering the number of roots per callus for 'OHxF 87', Figure 5, the treatment with greater result that differed statistically from the others, was the concentration of 16 mg L^{-1} of NAA in the culture medium, with tranversally leaf cutting (3.18 roots) followed by the lower concentration of 12 mg L^{-1} of NAA (2.34 roots). Regarding the presence of the two cytokinins studied, both were inferior to the presence of auxin, and regardless of the cutting direction of the explant, there were no significant differences between the different concentrations used. In this case, it could be necessary to balance the concentrations of cytokinin and auxin to stimulate callus formation, since isolated, high contents favor the development of aerial part and root system, respectively (George et al., 2008; Nakhooda et al., 2012).

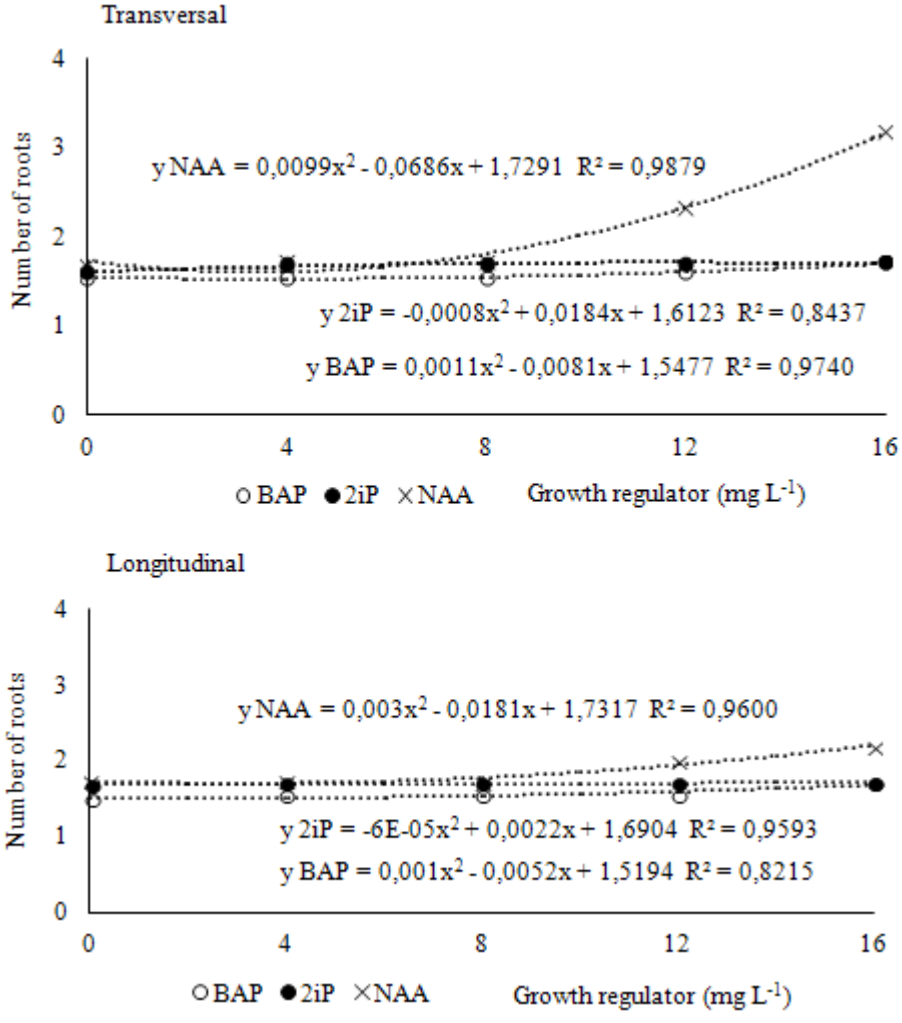

Figure 5 – Number of roots in relation to different growth regulators studied, their concentrations and leaf cuttings direction, during the *in vitro* morphogenesis of 'OHxF 87' explants.

The results obtained for BAP and 2iP in Figure 5, when compared with the use of auxin, are much lower for the variable number of roots, independently of the direction of the leaf cut, this can be justified by the fact of the cytokinin substances, such as BAP, hamper root meristematic activity, lateral root formation and act as an inhibitor when present at high concentrations in plants (Kozgar; Shahzad, 2012).

In the *in vitro* leaf morphogenesis of 'G 874' rootstock explants, the presence of NAA in the culture medium, as well as the transversal cutting direction, provided a higher number of roots, with a mean of 4.58 roots, with the concentration of 12 mg L^{-1} and when the cut did not maintain the leaf midrib (longitudinal), the concentrations that provided the highest occurrence of roots were 8 and 12 mg L^{-1} of NAA (Figure 6). For both cuts, transversely and longitudinally, the curve shows a negative behavior after the concentration of 12 mg L^{-1} of NAA, with a mean of 4.00 and 2.10 roots per callus, respectively. The importance of these results can be justified by Mitic et al. (2012), who attribute that the regeneration potential in apple explants, specifically 'Golden Delicious' and 'Melrose', needs optimization for each individual genotype, explant type and culture medium, as verified in this study, where differences were obtained for callus induction of 'G 874' in relation to presence and absence of the leaf midrib and in addition, the use of growth regulators in the culture medium.

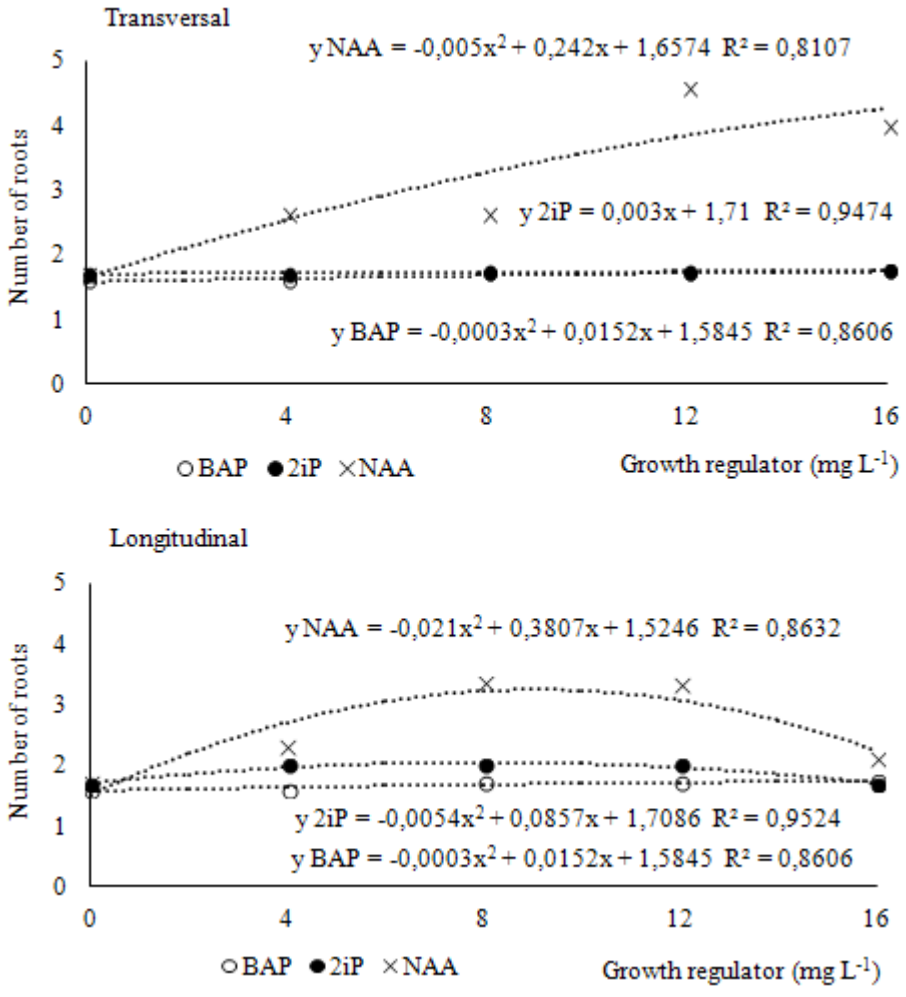

Figure 6 – Number of roots in relation to different growth regulators studied, their concentrations and leaf cutting direction, during the *in vitro* morphogenesis of 'G 874' explants.

It can be seen in Figure 6, that after the concentration of 8 mg L^{-1} of NAA there was a tendency of reduction of the roots number when the leaves were cut in the longitudinal direction. According to the results obtained in the present study, Kumar et al. (2016) conclude that as the concentration of auxin increases, the percentage of rooting tends to decrease for apple tree explants.

Figure 7 shows the root development in the calogenic explants of the cultivar OHxF 87, where once again, there was a triple interaction between the factors cutting directio x growth regulator x concentration, in this case, for the average root length variable.

Regardless of the leaf cutting direction, the concentration of 4 mg L^{-1} of NAA favored root elongation, followed by NAA concentration of 8 and 12 mg L^{-1}, where the non use of the regulator, as well the use of 16 mg L^{-1} of NAA provide roots of smaller length (between 0.8 and 1.03 cm). As observed in Figure 7, the use of auxin in the culture medium favored the development of the root system when compared to the use of two distinct cytokinins, BAP and 2iP, even at the highest concentrations, which is justified by Klerk et al. (2001) and Rolli et al. (2012), which explains that with the increase of these molecules in tissues, they become inhibitors of cell divisions.

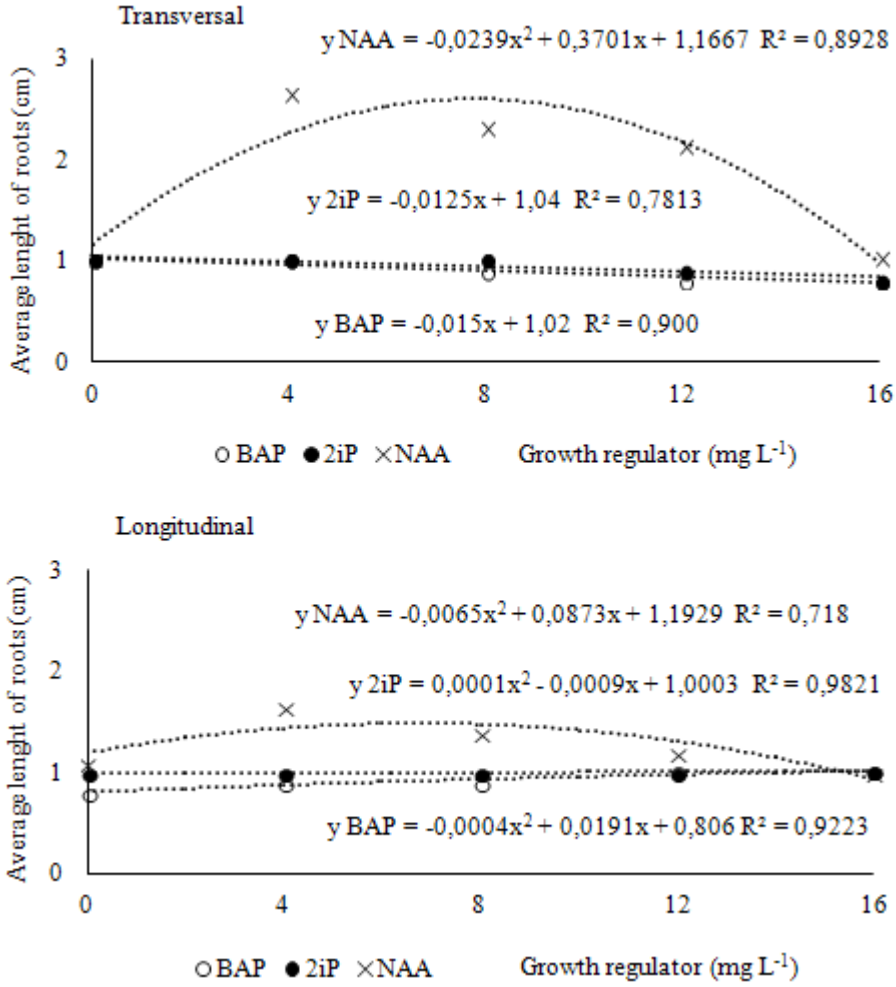

Figure 7 – Average root length (cm) in relation to different growth regulators studied, their concentrations and leaf cutting direction, during the *in vitro* morphogenesis of 'OHxF 87' explants.

In relation to the cutting of the leaves used as sources of initial explants, when were kept part of the leaf midrib, in the transverse cut, roots of longer length were obtained, with a mean of 2.65 cm in the concentration of 4 mg L^{-1} of NAA, while at the same dose, in the longitudinal cut, the roots reached an average length of 1.65 cm. Tang et al. (2002), in studies with sweet cherry (*Prunus avium* L.) and sour cherry (*Prunus cerasus* L.), in the same way, verified that the highest index of morphogenesis occurred from leaf midrib or in association with vascular tissue.

Similarly, occured for the same variable, with the 'G 874' rootstock, as shown in Figure 8. Comparing the growth regulators studied in both directions of leaf cutting, the cytokinins presented lower average root length results than when auxin was added to the culture medium. As for the cultivar OHx87, for 'G 874' when part of the leaf's midrib remained, there was formation of more elongated roots in the calluses originated from the morphogenesis, where the concentration of 8 mg L^{-1} of NAA provided roots of 2.45 cm and 2.10 cm, for the transverse and longitudinal cuts, respectively. When studying the influence of the explant type on explant regeneration, Silva et al. (2011) verified that regeneration in both MC and Adams cultivars occurred mainly in the basal region of the leaf explant, both via direct and indirect organogenesis. The same authors also point out that the entire leaf explant was more responsive to regeneration in relation to the use of basal thirds.

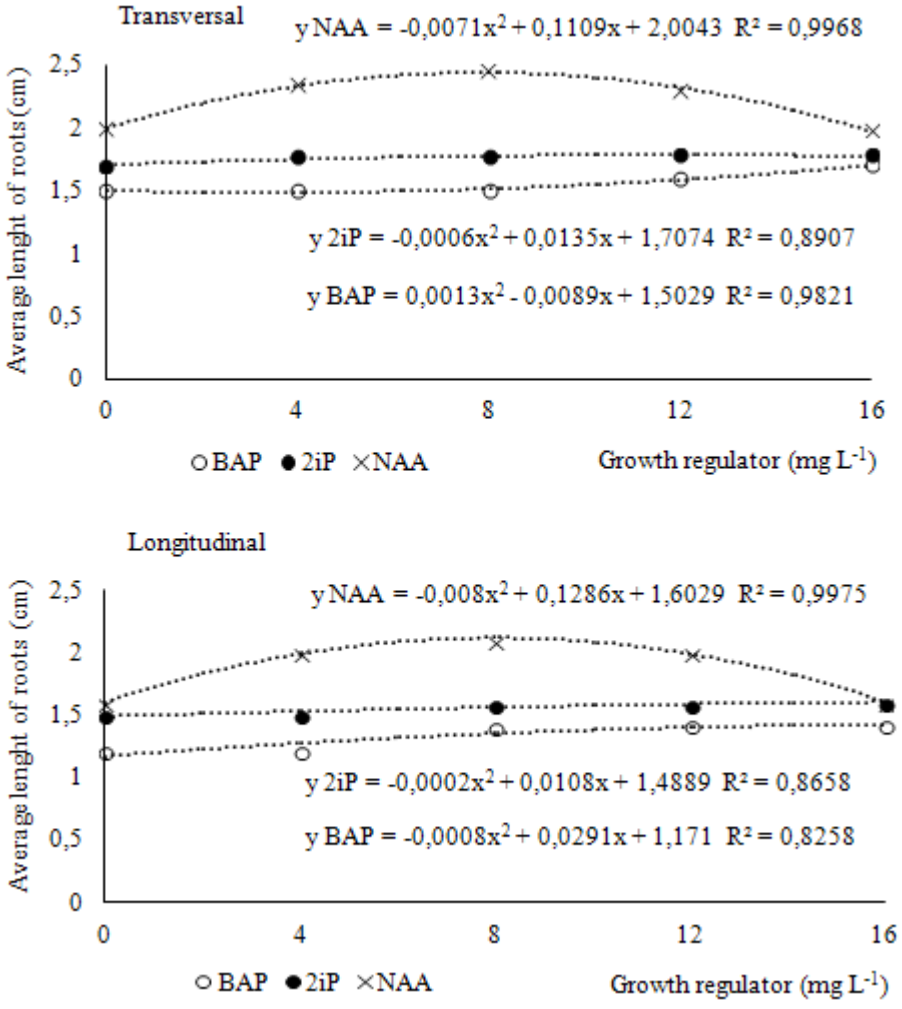

Figure 8 – Average length of roots (cm) in relation to different growth regulators studied, their concentrations and leaf cutting direction, during the *in vitro* morphogenesis of 'G 874' explants.

Kumar et al. (2016) when working with callus induction from 'Golden Delicious' apple tree leaves found that the roots reached a maximum length of 10.0 ± 0.57 cm at a concentration of 1 mg L^{-1} of AIB and also highlight the importance of root length increase, which results in a higher survival percentage of the explants.

In summary, according to Nogueira et al. (2007) and Botin and Carvalho (2015), callus induction is dependent on an intermediate hormonal balance of auxins and cytokinins, however, according to the results demonstrated in this research, it is verified that for the morphogenesis of the two rootstocks studied, 'OHxF 87' and 'G 874', the presence of auxin alone was sufficient for the calogenesis process of the explants for the two different cultivars. The same authors cited justify this theory from the possible balance between the auxin supplied in the culture medium and the endogenous content of cytokinins, since the initial source of explant already established *in vitro*, presented high juvenility.

CONCLUSIONS

The presence of the leaf's midrib favors the *in vitro* callus formation of 'OHxF 87' and 'G 874' rootstocks.

For induction of calluses in *in vitro* leaves of the rootstocks cv. OHxF 87 and cv. G 874 is required the presence of 4 mg L^{-1} of NAA and 8 mg L^{-1} of NAA, respectively.

The addition of auxin (ANA) to the culture medium favors *in vitro* morphogenesis of leaves, as well as the number of calluses and roots and root length of 'OHxF 87' and 'G 874' roots.

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