ISSN : 0974 - 7532 Volume 9 Issue 4

Research & Reviews in

BioSciences

Regular Paper

RRBS, 9(4), 2014 [122-133]

Evaluation of the dynamics of morphophysiological components during the adventitious rooting of *Eucalyptus nitens* and *Eucalyptus globulus*

María Paz Jofré[1]*, José Violido Becerra[2], Evelyn Daysi Bustos[2], Manuel Sanchez-Olate[1], Darcy Graciela Ríos[1]

[1]Laboratorio de Cultivo de Tejidos Vegetales, Facultad de Ciencias Forestales y Centro de Biotecnología, (CHILE)

[2]Laboratorio de Química de Recursos Naturales, Facultad de Ciencias Naturales y Oceanográficas, Universidad de Concepción, (CHILE)

E-mail : majofre@udec.cl

ABSTRACT

There are many events that occur during adventitious rooting of cuttings from a physiological and morphological point of view. In the current study we evaluated cuttings of *Eucalyptus nitens* and *Eucalyptus globulus* to determine the physiological and morphological conditions differing rooting capacity. For this we induced rooting by exogenous applications of 4000ppm of indol butyric acid (IBA). After 0, 2, 5, 15 and 30 days of induction we obtained samples at the base of the cuttings for the morphoanatomic and phytochemical analysis (total phenols, flavonoids) and indol acetic acid (IAA).

Morphoanatomical analysis shows that in both species root meristemoids formed from cells of the vascular cambium. During the rooting process there were differences between the two species on the measurements of phenolics compounds, E. nitens (800ug/gPF) had significantly higher values than E. globulus (200ug/gPF). Moreover, flavonoid compounds also differ between species, where rutin and quercetin were present only in E. nitens. This indicate that the phenolics compounds could make of difference. During the induction phase (first two days), IAA was in high concentrations, which decreased drastically during the following phases of adventitious rooting, this suggests a fundamental role in the induction phase of adventitious rhizogenesis.

KEYWORDS

Adventitious rooting;
Flavonoids;
Morphoanatomy;
Indol acetic acid;
Phenols.

INTRODUCTION

The vegetative production of forest species using in vitro micropropagation and macropropagation techniques is of great interest because of its potential use in research and plant production management. This allows to maintain the genetic gain developed by traditional improvement programs and take them to the plantation as value added for wood production[1,2]. In vitro propagation methods in Eucalyptus have been successful because it preserves plant genetic homogeneity; this method has been widely used in New Zeland, Portugal, Spain, Brazil, Australia, South Africa and Chile[1,3]. This technique is commonly used in horticulture, floriculture, forestry and for conventional enhancement[4]. However, in recalcitrant species there have been diffi-

culties in the adventitious rooting of cuttings[5].

Adventitious rooting is an essential step in the propagation of tree cuttings of select genotypes. Roots originate from the dedifferentiated parenchyma surrounding the vascular tissues due to the action of IAA[6]. The cortical parenchyma formation and the interruption of the stem vascular cylinder allows the communication between both parenchymas (pith and bark) which originates the root meristem. However, the pith parenchyma has dividing cells and cells with silica or calcium oxalate crystals that provide calcium for cellular growth[7]. The root originates from meristematic nodules located in the surrounding area of the vascular cambium, the cells divide in one area in a polarized form[8]. These nodules initiate from the activity of the vascular cambium, forming zones with alternate cellular proliferations that coincide with the pith radius. This formation increases the radial parenchyma that pushes the phloem and periphloematicfibre nodules towards the bark[8]. In this context auxins play a fundamental role in adventitious root formation where there is a positive correlation between the endogenous levels of IAA and its ability as root inducer[9].

Exogenous levels of IBA inhibit the elongation of the main root and induce the formation of lateral and adventitious roots[10]. This response is due to IBA is converted to IAA in the peroxisome through β-oxidation[9,11,12] promoting a low delivery and efficiency, because it acts to minimal inhibitory levels for root elongation[10]. The factors that control free auxin levels are peroxidase with IAA oxidase activity (IAA-ox), polar transport, the conjugation of esters formation or esters among auxins and sugars or amino acids[9,13-15].

Phenolic compounds are known to be defense factors against various types of stresses caused by pathogens, adverse environment or injury[16]. These compounds also play an important role in the formation and growth of adventitious roots because of its control of IAA. Compound regulation depends on the chemical nature, polyphenols would be protecting against IAA destruction and the monophenols oxidizing IAA because of the estimulation of IAA-ox[4].

Another important group of regulatory molecules are flavonoids, which are located throughout the plant and they are involved in many physiological processes such as the transport of auxins, plant pigment, pollen germination, and biotic stress signaling[17,18]. The role of flavonoids as inhibitors of auxin is evident when mutating the gene chalcone synthase, a key enzyme in the biosynthetic path of flavonoids. When the gene chalcone synthase is mutated, plant growth and development is stimulated[19-22]. Mutants of Arabidopsis have abundance of mRNA that code for the genes in the protein family PIN where the main function is to allow the transport of auxins in the plant[21,23] Lazar y Goodman, 2006). Enzymes of flavonoid biosynthesis accumulate in the nucleus[24], in areas where bud and root meet[19,25],it is in this area where the movement, distribution and localization of auxins are blocked. These are competing with endogenous inhibitors of polar transportation, by ways of coupling or blocking facilitating proteins of auxin efflux[19,21].

Based on the previous evidence, we will evaluate the morphoanatomy, IAA contents, phenolic and flavonoid compounds during the rhyzogenic process of Eucalyptusnitensand *Eucalyptus globulus*. To establish its relationship with low rooting capacity of E. nitens and may serve as a marker of adventitious rooting.

MATERIALS AND METHODS

Plant material

Donor plant clones of *Eucalyptus nitens* and E. globulus from the nursery Forestal Mininco S.A. in Los Ángeles, Chile were used as a source of cuttings. These cuttings were maintained in cultivating conditions in a regulated watering regime, relative humidity, temperature and nutrition and phytosanitary conditions.

Root induction

Cuttings were induced with indol butyric acid (AIB, 4000 ppm) in the cut area. After treatment the cuttings were maintained under productive conditions for 45 days. During this time we collected samples of the treated cuttings at 2, 5, 15 and 30 days of cultivation for morpho-anatomical and phytochemical analysis. We took samples at time zero (0) of the cuttings before the AIB treatment.

Morpho-anatomical analysis

We collected pieces of tissue from the base of the cuttings. These were immediately fixed in formaldehyde acetic acid alcohol (F.A.A.) for 72 hours and were then transferred to ethanol 70% (v/v) for morpho-anatomi-

Regular Paper

cal analysis. We obtained histological cuts at the base of the cutting with a microtome blade. The cuts were analyzed under a microscope Olympus coupled with the software Micrometric S.E. Premium.

Determination of IAA

From the base of the cuttings collected at 0, 2, 5, 15 and 30 days, we extracted the hormone IAA following the protocol by Valenzuela et al. (1998). We extracted and separated the organic phase that contains the free form of IAA and the aqueous phase that contains the conjugated form of IAA. The organic phase was resuspended in absolute methanol to be analyzed in HPLC Shimadzu, Kyoto, Japan. The HPLC was controlled by the software D 7000 HPLC, with a pump D7200 and detector UV L /7400, separation was carried out by HPLC RP-18 reverse phase 250-4mm (5um) Bio Rad in a column Lychrospher 100 at 0.8ml/min. Identification of IAA was obtained by comparing the retention time of the sample against a standard IAA, Sigma.

Determination of total phenols

From the base of the cuttings collected at 0, 2, 5, 15 and 30 days, these samples were immersed in methanol 80% for 24 hours in dark conditions. The sample extracts were obtained and evaluated for total phenols, which were estimated as thegalic acid equivalents according to the modified method by Dastmalchi. This method uses the Folin-Ciocalteu reagent (oxidizing agent) and it is quantified by spectrophotometry (760nm) based on the colorimetric reaction of reduction-oxidation. This method determines the total phenol concentration by extrapolating the standard galic acid curve.

Determination of flavonoids

From the extracts obtained in section 2.5, we determined specific flavonoids through HPLC Shimadzu, Kyoto, Japan. The HPLC was controlled by the software D 7000, equipped with a column RP-18 reverse phase 150-4.6 mm (5um) Lychrospher. The first 15 minutes the flux is maintained at 0.7 ml/min for the extract, between 15-25 minutes the extract is replaced witha solution of 40% water and 60% acetonitrile, after 25 minutes it is replaced with a solution of 20% water and 80% acetonitrile. Flavonoids were determined comparing the retention of flavonoid standards such as quercetine, rutin, luteoline, caffeic acid and ferulic acid.

Statisrical analysis

Statistical analysis was performed with mixed linear statistical models to address the lack of any of the classical assumptions requiring traditional variance analysis. For analysis of the variables and AIA, phenol concentration was analyzed by repeated measurements to longitudinal analysis. In the case of the phenol concentration were considered five measurements over time (days 0, 2, 5, 15 and 30); and in the case of IAA concentration were considered only two measurements in time (day 0 and 2), as for the other measurements, concentrations were 0 and adjustment models did not converge, this analysis allows to identify both the average treatment effect (species), the effect of time (day) and the time by treatment interaction, which correspond to three hypotheses tested, and therefore generate three values p. This models included species associations, time and interaction effects in the mean structure (fixed effects), several structures for the residual variance - co variance matrix were proved.

Parameter estimation was done by restricted maximum likelihood (REML). Model selection was done with Akaike information criterion (smaller is better). Models were estimated using SAS PROCMIXED version 9.3 (SAS Institute, 2006). Pairwise comparisons between species associations were tested with the adjusted LSMeans (α =0.05).

RESULTS AND DISCUSSION

Morpho-anatomical analysis

Under normal conditions (day 0) both E. globulus and E. nitens (Figure 1a and Figure 2a) have an anatomical structure made of an external epidermis, bark made of parenchymal cells, a vascular system surrounding the pith, the xylem cells are located near the pith to the vascular cambium, and the phloem is a ring of continuous parenchymal and schlerenchymal cells extending towards the bark area.

After root induction occurred on day 15, it is possible to find a disorganized area (thickening) that might correspond to the generation of new cells. This area allows for the dissapearence of the square structure with pronounced verticesin both Eucalyptus (Figure 1c and Figure 2c).

Regular Paper

In a previous study it was reported that the rooting process in vitro of E. globulus it was possible to observe changes from day 7 after root induction[26]. Such changes were disorganized cells, abundant cells going through mitosis, specially small cells in the cortical parenchyma that have prominent nuclei[27].

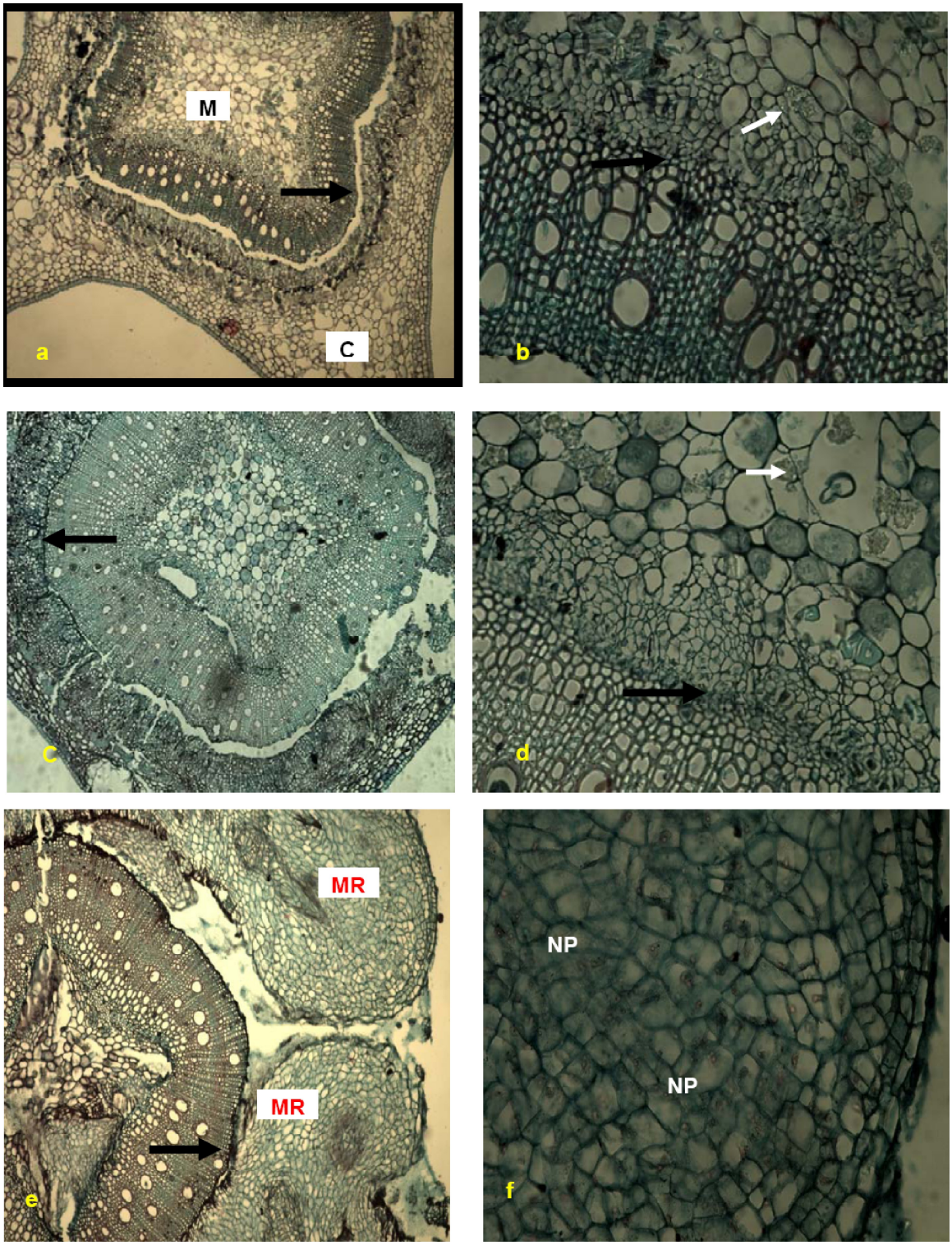

Figure 1 : Tissue analysis at the base of cuttings from *Eucalyptus nitens*: a and b) day 0 of induction the parenchymal tissue is forming the pith (M), bark (C), and vascular cambium shown by black arrow. (c and d) Day 15 of induction, (c) shown is the loss of the square structure of day 0, black arrow shows vascular cambium (e) white arrow shows crystals in between parenchymal cells and black arrow shows vascular cambium. (e and f) Day 30 of induction, presence of root meristems (MR) (f) isodiametric cells of prominent nuclei (NP).

Regular Paper

At day 30 after root induction we can observe the formation of root meristems (MR) with cells in division and prominent nuclei (NP) that correspond to the dedifferentiation zone (Figure 1e y Figure 2e). This dedifferentiation zone (MR) originates from the parenchymal cells near the vascular cambium and cortical parenchyma. At this stage the cortical parenchyma completely deforms because of cellular division that will allow the emergence of a new root[6]. In an anatomical study of Alnusglutinosathe meristematic zone is charac-

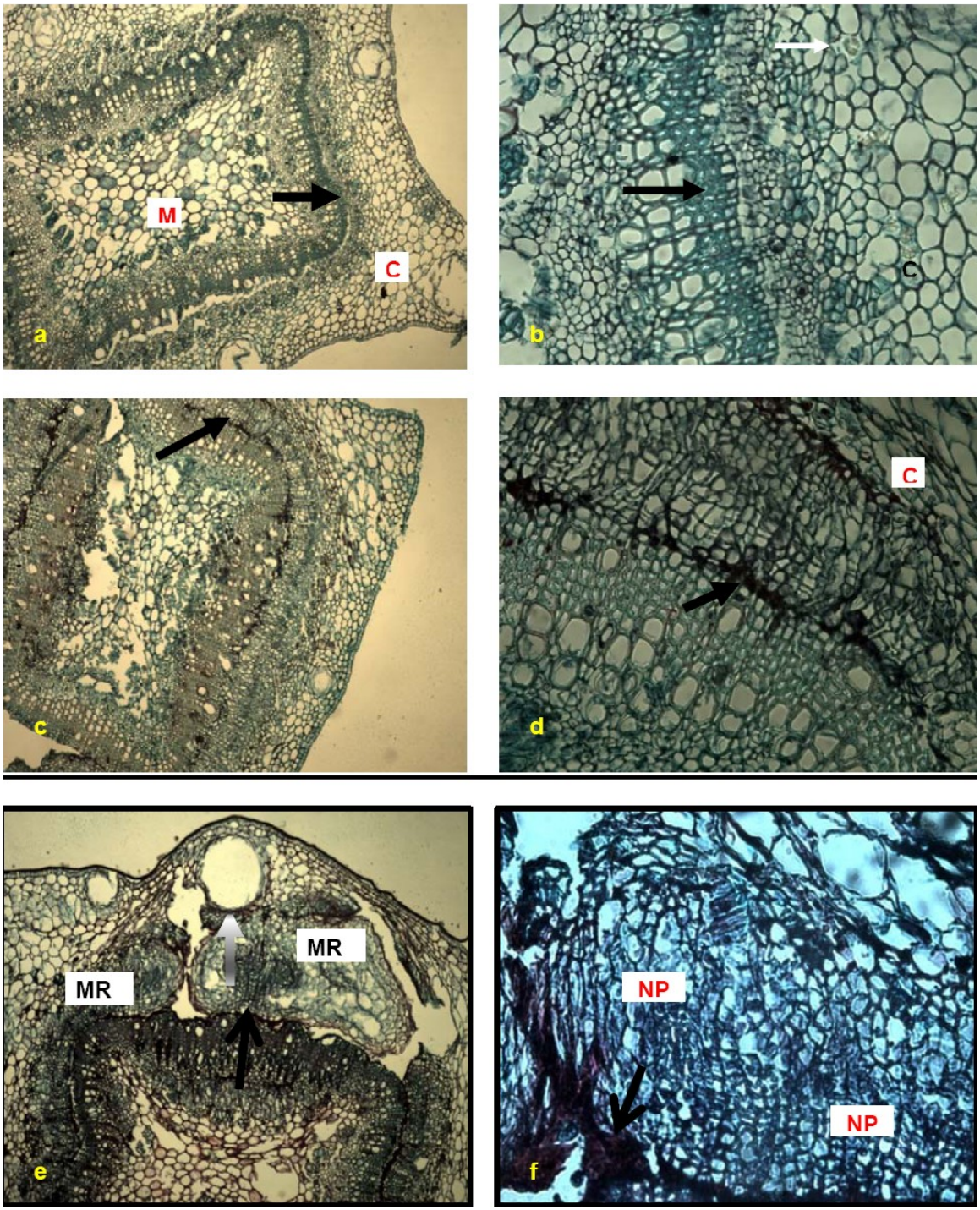

Figure 2 : Tissue analysis at the base of cuttings from *Eucalyptus globulus*: (*a and b)* day 0 of induction shown is the Pith (M) and Bark (C), black arrow shows the vascular cambium. In *(b) and (d)* white arrow shows crystals in between cortical parenchymal. (*c and d)* Day 15 of induction, black arrow shows vascular cambium. (*e and f)* day 30 of induction, shows presence of root meristems *(MR)* forming from the vascular cambium black arrow, and *(f)* isodiametric cells of prominent nuclei (*NP)*.

terized by prominent nuclei and nucleoli because of its active mitotic division[28]. In the current study with Eucalyptus we observed similar patterns in both species (Figure 1f y Figure 2f). The formation of the root meristem is direct because there is no formation of a callus at the base of the cutting; this can have the advantage because the formation of the tissue can interfere in the vascular connection that is later established between the new root and the original explant[28,29].

Because of the dedifferentiation nature of the root meristem, it can correspond to the quiescent center reported by[30], which can later be induced by auxin to become differentiated. This induced differentiation can be done to individualize the original primordial tissue and form the structures of a new adventitious root. However, it is important to note that the root primordial does not necessarily originate a root[28].

The anatomy found at day 30 after induction in both Eucalyptus species is similar to those observed in in vitro rooting for other species such as apples[31] and chestnut[32] and after 18 days in E. globulus[26]. However, the time needed to form adventitious roots depends on the species. For example, in Camellia japonica adventitious roots formed after 8 days[31,33], but in both cases the root development was faster in in vitro compared to conventional conditions.

We also observed abundant crystals in between cortical parenchyma cells. This crystal formation is probably calcium oxalate that act as messengers during the auxinic action of root development[26] also found crystals of octagonal and pyramid shape in E. globulus; however, their nature was not determined.

In the current study we did not find differences in the morpho-anatomy at the base of the cuttings in either species of Eucalyptus. However, in Enebus cretica there are genotypes with different rooting capacity that have anatomical differences[34].

Determination of AIA

We observed high levels of IAA in the first two days of induction and then the levels drastically decreased the following days (TABLE 1). This may indicate that the high concentration levels of IAA can correspond to the induction phase, and the lower concentrations may indicate the initial phase. Similar results were also found by[35-37]. Another study found higher levels of IAA on the third day of root induction and a decrease on day 6 in Vignaradiata[38].

The increase of IAA on the days of root induction can be related to a low activity of oxidase; on the other hand, the subsequent decrease of IAA can be related to an increased activity of the enzyme IAA oxidase (IAAox) and the conjugates of IAA[36,39]. During the inductive phase, IAA controls the cell division and expansion, initiation of lateral roots, and stem and root elongation[40].

There are various mechanisms that make auxin available for competent cells. Among these are: its biosynthesis, peroxidase with IAAox activity[14,15], its polar transport[41] and its conjugation in the form of esters or amides among auxins and sugars or amino acids[9,42].

Studies have reported that the increase in IAAox activity on the first days after root induction can be a response to the application of exogenous auxin to control the excess of free auxin. The apoplastic peroxidase acidify surrounding tissue which allow signaling of auxinic action at the membrane level for H^+ pumping. This action results in cell wall plasticity in the intitial phase for cell elongation and growth[43].

On the other hand[44] suggests that exogenous auxinsbond to proteins; therefore, auxins will have a prosthetic group that regulates endogenous levels of free

TABLE 1: Concentration of AIA at the base of the cuttings of *Eucalyptus nitens* and *Eucalyptus globulus* during rizhogenesis. At below of the table statistical analysis indicating significant differences between species, interaction time between the two

Días de colecta	Conc. AIA ug/gPS *E.nitens*	Conc. AIA ug/gPS *E.globulus*
0	702 a	543 b
2	647 a	723 b
5	0	0
15	0	0
30	0	0

Tratamiento	Tratamiento	Estimación	Error Estándar	GL	Valor t	Pr > |t|
Globulus	Nitens	-1852.93	25.2040	16	-73.52	<.0001

Regular Paper

auxin.In fact, there are hydrolases that facilitate the decoupling of IAA in certain tissues[45]. For example, enzymes of the following families: MtlAR31, MtlAR32, MtlAR33 and MtlAR31 have hydrolytic action over IAA-aspartate and IBA-alanine[46]. These hydrolase enzymes are highly conserved, and are found in many monocotyledon and gymnosperms[45,47-49].

Another explanation of the high content of IAA during root induction can be related to the exogenous application of IBA. It has been reported that IBA could be inducing the basipetal movement of endogenous IAA, as reported by[50]. This would allow a continuous source by means of the slow transformation of IBA to IAA. Moreover, there is genetic evidence in Arabidopsis thaliana that the auxinic IBA precursor is converted to active IAA by β oxidation at the peroxisome level[51,52] evaluated IBA mutants resistant and sensitive to IAA, the phenotype had short root hair and small cotyledons. It was possible to mend this phenotype with exogenous applications of IBA, this demonstrates its modulator role on IAA[51], providing it chemical stability[36,42,53]. An effective response to the application of exogenous IBA and its conversion to IAA depends on the genes involved in the enzyme synthesis that participate in the conversion process[52], genes such as PEROXIN 4 code for enzymes that reside in the peroxisome[54].

In the current study we observed that both Eucalyptus species have similar dynamics of IAA contents (TABLE 1). We were able to detect high levels of IAA during the first days of root induction, and during these days we also found high levels of flavonoids[20]. Furthermore, found high levels of flavonoids in Arabidopsis thalianathat act as endogenous inhibitors of the polar transport of auxins.

Making it possible to find that despite a high concentration of free auxin during rooting induction auxin is not necessarily active since its function is largely provided by a signaling cascade that begins with the first direction and reprogramming cellular given efflux proteins of IAA, PIN proteins[9,55,56].

Determination of phenolic compounds

We measured the total concentration of total phenols and found that E. nitens has significantly higher concentration than E. globulus (TABLE 2, Figure 3). Moreover, for E. nitensthe concentrations of these compounds differ in each phase (induction, differentiation, elongation, and emergence) as described by[57,58]. There was a maximum concentration reached in the induction phase of rhizogenesis, which is between 0 and 2 days. This maximum was followed by a significant decrease on day 5, and an increase of total phenol concentration on days 15 and 30.

Phenolic compounds inhibit oxidation of IAA because they have antioxidant capacity, which makes them rooting cofactors. On the other hand, there are rooting inhibitors that contribute to the oxidation of IAA[59,60].

The high level of phenolic compounds in E.nitens may explain the low rooting capacity. Possibly there may be monophenols that act as inhibitors of the rhizogenic process, reducing the IAA concentration. According to[60] certain monophenols such as p-coumeric, ferulic and syringic acid promote decarboxylation through the enzyme IAA oxidase.

Studies on Nothofagus pumilio suggest that high concentration of total soluble phenolic compounds would be inducing oxidation to the applied auxin (IBA) to the rooting treatment. These soluble phenols can be rooting inhibitors depending on quality and quantity[61].

Phenolic compound content in E. globulus did not

TABLE 2 : Total phenols concentration at the base of cuttings of *Eucalyptus nitens* and *Eucalyptus globulus* during rhizogenesis. Diffferent letters indicate significant differences. At the foot of the table statistical analysis indicating significant differences between species, einteracción time between the two

Day of collection	*Eucalyptus nitens* ug/gPS	*Eucalyptus globules* ug/gPS
0	136 a	29 b
2	130 a	23 b
5	53 b	36 b
15	109 a	31 b
30	102 a	33 b

Tratamiento	Tratamiento	Estimación	Error Estándar	GL	Valor t	Pr > |t|
Globulus	Nitens	- 525.32	17.2481	31.6	- 30.46	<.0001

Regular Paper

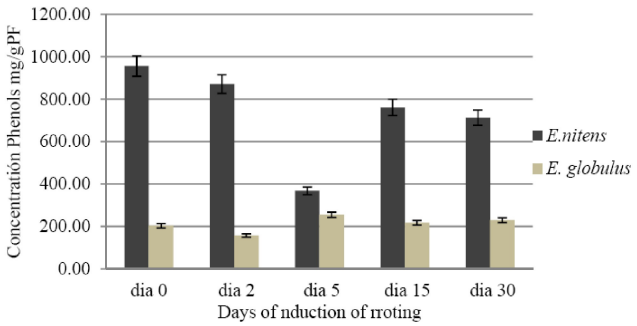

Figure 3 : Total phenolic compounds from the base of cuttings in *Eucalyptus nitens* and *Eucalyptus globulus* during the rhizogenic process (0,2,5,15 and 20 after the induction of rooting)

differ among collecting days. The amount of phenolic compounds is much lower than E. nitens, which can be favorable for the rooting process if there are inhibitors such as monophenols.

Moreover, if the phenolic compounds were polyphenols then they would be inducing rooting. There are reports that many of these such as chlorogenic, protocateuchico and caffeic acids suppress the degradation of IAA because they activate the enzyme polyphenol oxidase which are oxidized to quinones and they would not be able to bond or estimulate the enzyme IAA oxidase[60].

TABLE 3 : Flavonoid concentration (ug/g) from extracts obtained from cutting bases of *Eucalyptus nitens* (N) and *E. globulus* (G) for the different sampling days. N.D. indica que no fue detectado por HPLC

Muestra	Rutin ug/g	Quercitrina ug/g	Ac.Felúrico ug/g	Ac.Caféico ug/g	Luteolina ug/g
NO	11.8	0.3	N.D.	0.3	0
N2	5.8	0.1	N.D.	0	0
N5	9.7	0	N.D.	0.5	0
N15	3.5	0.2	N.D.	0.6	0
N30	0	0.4	N.D.	0	0
GO	0	0	N.D.	0	0
G2	0	0	N.D.	1	0.6
G5	0	0	N.D.	0.2	0.6
G15	0.3	0	N.D.	0	0
G30	26.4	0	N.D.	0	0

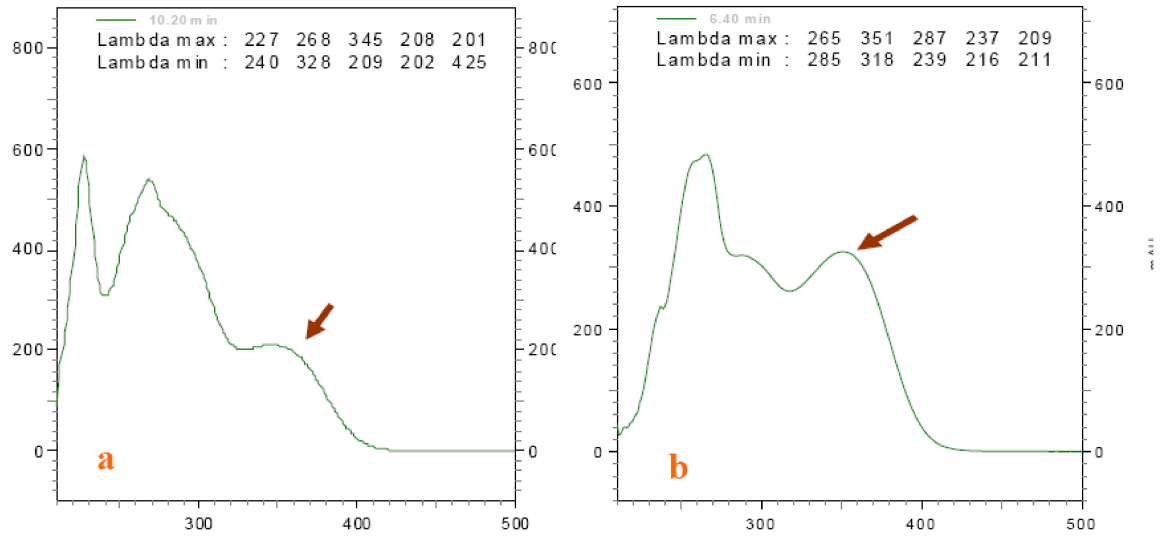

Figure 4 : Flavonoid spectrums obtained from *Eucalyptus nitens* cuttings in the process of rooting at day 5 for a) kaempherol, time 6.4 min, 364.5 nm; b) rutin, time 10.2 min. 350nm

Based on the latter, we can impart that phenolic compounds have an important role controlling rooting in species with low rhizogenic capacity; however, they do not seem to be as important in species with higher rhizogenic capacity such as in E. globulus. For other Eucalyptus species there are genotypes with high rhizogenic capacity where the maximum total phenols are manifested on day 1 after induction and then decrease drastically on day 2. However, in genotypes with low rooting capacity have a low and stable amount of

Regular Paper

phenols during the rooting process[59].

Determination of flavonoids

We detectedrutin during the whole rooting process in E. nitens except on day 30. We observed the opposite trend in E. globulus, rutin is present only at the end of the rooting process, specifically on days 15 and 30; on day 30 we observe a significantly higher value for rutin.

Quercetin was present in concentrations that are significantly different between the two Eucalyptus, which makes it an excellent marker candidate for the rhizogenic process. Its presence in E. nitens indicates rooting inhibition since there is evidence of regulating auxin, by inhibiting the aminopeptidase acitivity in vivo[19,25]. In our study, quercetin is present on the first days of induction, disappearing on day 5 and reappearing on days 15 and 30 for E. nitens. We did not detect any quercitin in E. globulus.

Eucalyptus globulu srutin dynamics is opposite. The dynamics of quercitin and rutin can explain the rooting difficulties of Eucalyptus. In fact, other studies suggest that quercitin and kaempferol are specific members of the chemical family that inhibit the transport of auxins[25]. Quercetin would be a competitive inhibitor of a protein transporter of auxin[19] of similar structure to naphthylphthalamic acid (NPA)[20].

Figure 4 shows the flavonoid spectrum of kaempherol and rutin from E. nitens on day 5. These flavonols have been directly related with the homeostasis of IAA because of their presence during rooting and its transport from cell to cell[62]. There is also evidence that certain transporting protein of IAA marked by flavonoids (PGP1) is sensitive to be inhibited by quercetin[63]. Flavonoids are not only known to regulate the polar transport of auxin and ethylene signaling[20,21,64-66], but are also known to modulate IAAox activity maintaining the homeostatic control of auxin[67].

There are specific interactions between flavonoids and facilitating proteins for auxin efflux (PIN)[19,25]. Auxin moves basipetally throughout the vascular system and accumulate in the progenitor cells in the root. Flavonoid transport is similar to auxin transport whereby long distance transport is ATP-binding cassette (ABC)[68-70] and is competing for substrate with IAA[71,72]. Flavonoids have a similar chemical function to that of the competitive endogenous inhibitor NPA. Its synthesis depends on the environment, and its location and distribution is related to auxins[20]. On the other hand, ethylene produces the asymmetric accumulation of flavonoids, impeding the transport of IAA[63,66,72].

Moreover, ethylene, which may be biosynthesized by the stress produced by the cutting injury, results in the accumulation of flavonoids asymmetric preventing transport AIA[63,66,72].

Finally, there is evidence that in the absence of flavonoids there is an increase in mRNA transcription for PIN proteins that facilitate polar transport of auxins[25].

ABBREVIATIOS

AIA : Acido Indol Acético
IBA : Acido Indol Butírico
EA : Enraizamiento Adventicio

CONCLUSION

The adventitious rooting in both species of Eucaluptus no significant differences in morphoanatomy. The formation of a root meristem begins from the parenchymal cells near the vascular cambium and it is evident that at day 30 of rooting with IBA. Also there is a high concentration of IAA during induction, but this IAA concentration decreases to undetectable concentrations in the following stages of rhizogenesis.

Concentration of phenolic compounds is significantly higher in E. nitens compared to E. globulus. These compounds are possibly acting as inhibitors of rooting. This difference could be explaining the low rooting capacity of E. nitens. Since, specific flavonoids of the type flavonols were in high concentrations in E. nitens. These compounds are known to have a negative regulatory role in the transport of auxins, which indicate the relationship of these compounds with the low rooting capacity of E. nitens.

ACKNOWLEDGEMENTS

We would like to thank Comisión Nacional de Investigación Científica y Tecnológica, CONICYT for funding the Doctorate degree and thesis, and project 1020F11 INNOVA BioBio. We are also very grateful for the collaboration of Forestal Mininco S.A. for providing plant material for this study. And finally we would

like to thank Dr. Ingrid Aguayo for reviewing and translating the current publication.

REFERENCES

[1] M.Campbell, A.Brunner, H.Jones, S.Strauss; Forestry is fertilecrescent the application of biotechnology to forest trees. Plant.Biotechnol.J., **1**, 141-154 **(2003)**.

[2] K.Delaporte, M.Sedgley; Breeding of Eucalypt bud and flower lines A report for the Rural Industries Research and Development Corporation.Cap. Propagación clonal, 22-33 **(2004)**.

[3] M.J.Gaspar, N.Borralho, A.Lopes; Comparison between field performance of cuttings and seedlings of *Eucalyptus globulus*. Ann.Sci., **62**, 837-841 **(2005)**.

[4] H.T.Hartmann, D.E.Kester, F.T.Davies, R.L.Geneve; Hartmann and Kester's Plant Propagation: Principles and Practice, 7th Edition. Prentice Hall, Upper Saddle River, New Jersey, **(2002)**.

[5] A.Fett-Neto, J.Fett, L.Vieira, G.Pascuali, R.Termignomi, Ferreira; Distincseffects of auxin on adventitious root development Eucalyptus saligna and *Eucalyptus globulus*. Tree Phys., **21**, 437-461 **(2001)**.

[6] M.S.Soh, S.H.Hong, B.C.Kim, J.Vizir, D.H.Park, G.Choi, M.Y.Hong, G.Y.Y.Chun, M.Furuya, H.G.Nam; Regulation of both light and auxin-mediateddevelopment by the Arabidopsis IAA3/ SHY2 gene. Journal of Plant Biology, **42**, 239-246 **(1998)**.

[7] Strasburger; Tratado de Botánica.Editorial Omega ISBN-10: 8428213534 ISBN-13: 978-8428213530, **(2004)**.

[8] L.Laskowski, D.Bautista; Características anatómicas de raíces adventicias en estacas de semeruco (Malpighi emarginata (DC) tratadas con Acidoindol butírico. Bioagro, **11(3)**, 88-96 **(1999)**.

[9] A.Woodward, B.Bartel; Auxin: Regulation, Action and Interaction. Annals of Botany, **95**, 707-735 **(2005)**.

[10] B.K.Zolman, B.Bartel; Identificacion y caracterización de ArabidopsisIndol-butírico mutantes defectuosos de respuesta de las enzimas peroxisomales. Genética, **180**, 237-251 **(2008)**.

[11] J.Poupart, M.Wadell; Transporte de las dos auxinas naturales Ácido-3-Indol- Butírico y Acido-3-Indol-Acético, en Arabidopsisthaliana. Physiology. Vegetal, **133(2)**, 761-772 **(2000)**.

[12] L.Strader, G.Chen, B.Bartel; Ethylene directs auxin control root cellexpansion. The Plant Journal, **64**, 874-884 **(2010)**.

[13] J.Friml; Auxin transport – zapping the plant. Current Opinion Plant Biology, **6**, 7-12 **(2003)**.

[14] M.Mc Donald, J.Wynne; Adventitious Root Formation in Woody Tissue: Peroxidase – A Predictive Marker of Root Induction in Betulapendula. In Vitro Cell Des.Biology-Plant, **39**, 234-235 **(2003)**.

[15] D.J.Metaxas, T.D.Syros, T.Yupsanis, A.S.Economou; Peroxidases during adventitious rooting in cuttings of Arbutus unedo and Taxusboccata as affected by plant genotype and growth regulator treatment. Plant Growth Reg., **44**, 257-266 **(2004)**.

[16] A.Solar, M.Colaric, V.Usenik, F.Stampar; Seasonal variations of selected flavonoids, phenolc acids and quinons in annual shoots of common walnut (Juglans regiaL.). PlantSci., **170**, 453-461 **(2006)**.

[17] R.Koes, W.Verweij, F.Quanttrocchio; Flavonoides: un modelo colorimétrico de la regulación y la evolución de las vías bioquímicas. Plant Science, **10(5)**, 236-242 **(2005)**.

[18] L.P.Taylor, E.Grotewold; Flavonoids as developmental regulators. Curr.Opin.Plant.Biol., **8**, 317-323 **(2005)**.

[19] A.Murphy, W.A.Peer, L.Taíz, Regulation of auxin transport by aminopeptidases and endogenous flavonoids. Planta, **211**, 315-324 **(2000)**.

[20] D.Brown, A.Rashotte, A.Murphy, J.Normanly, B.Tague, W.Peer, L.Taiz, G.Muday; Flavonoides Act as Negative Regulators of Auxin Transport in Vivo in Arabidopsis. Plant Physiology, **126**, 524-535 **(2001)**.

[21] W.Peer, A.Bandyopadhyay, J.Blakeslee, S.Makam, R.Chen, P.Masson, A.Murphy; Variation in Expression and Protein Localization of the PIN Family of Auxin Efflux Facilitator Proteins in Flavonoid Mutants with Altered Auxin Transport in Arabidopsis thaliana. The Plant Cell, **16**, 1898-1911 **(2004)**.

[22] C.Buer, G.Muday, M.Djordjevic; Flavonoids are differentially Taken Up and Transported Long Distances in Arabidopsis. Plan Physiology, **145**, 478-490 **(2007)**.

[23] G.Lazar, H.M.Goodman; MAX1, un regulador de la vía de Flavonoides, control vegetativo consecuencia en la yema axilar de Arabidopsis. Proc.Nat.Acad.Sci., **103(2)**, 472-476 **(2006)**.

[24] D.E.Saslowsky, U.Warek, B.S.Winkel; Nuclear localization of flavonoid enzymes in Arabidopsis. J.Biol.Chem., **280**, 23735–23740 **(2005)**.

[25] W.A.Peer, D.E.Brown, B.W.Tague, G.K.Muday, L.Taiz, A.S.Murphy; Flavonoid accumulation patterns of transparent testa mutants of Arabidopsis. Plant.Physiol., **126**, 536–548 **(2001)**.

Regular Paper

[26] X.Calderón, G.Montenegro, E.García; Ontogeny of in Vitro rooting processes in *Eucalyptus globulus*. In vitro Cell Development Biology Plant., **40**, 499-508 **(2004)**.

[27] D.Reinhard, T.Mandel, C.Kuhlemeier; Auxin Regulates the initiation and Radial position of Plant Lateral Organs. The Plant Cell, **12**, 507-518 **(2000)**.

[28] M.C.San José, L.Romero, L.Janeiro; Estudio anatómico del desarrollo de raíces adventicias en microestaquillas de Alnus glutinosa (L.) Gaertn Revista Real Academia Galega, **31**, 15-26 **(2012)**.

[29] G.Martínez-Pastur, M.Arena, L.Hernández, N.Curvetto, E.Eliasco; Histological events during in vitro rooting of Notofagus nervosa (Fagaceae). N.Z.J.Bot., **43**, 61-70 **(2005)**.

[30] K.Xu, X.Xu, F.Takeshi, P.Canlas, R.Maghirang-Rodríguez; Sub1A es un gen de etileno-respuesta como factor que confiere tolerancia a la sumersión de arroz. Naturaleza, **442**, 705-708 **(2006)**.

[31] S.Naija, N.Elloumi, N.Jbir, S.Ammar, C.Kevers; Anatomical and biochemical changes during adventitious rooting of apple rootstocks MM 106 cultured in vitro. C.R.Biologies, **331**, 518-525 **(2008)**.

[32] A.Ballester, N.Vidal, A.M.Vieitez; Developmental stages during in vitro rooting of hardwoods trees with juvenile and mature characteristics. En K.Niemi, C.Scagel; (Eds); Adventitious Root Formation of Forest Trees and Horticultural Plants-from Genes to Applications. Research Signpost, Kerala, India, 277-296 **(2009)**.

[33] A.Samartin, A.M.Vieitez, E.Vieitez; Rooting of tissue cultured camellias, J.Hortic.Sci., **61**, 113–120 **(1986)**.

[34] T.Syros, T.Yupsanis, H.Zafiriadis, A.Economou; Activity and isoforms of peroxidases, lignin and anatomy, during adventitious rooting in cuttings of Ebenus creticaL. J.Plant.Physiol., **161**, 69–77 **(2004)**.

[35] T.Gaspar, M.Hofinzer; Auxin metabolism during adventitious rooting.In: T.D.Davis, B.L.Haissig, N.Sankhla, (Eds); Adventitious root formation in cuttings. Portland: Dioscorides Press, 117-131 **(1988)**.

[36] S.Nag, K.Saha, M.A.Chouthuri; Role of Auxin and Polyamines in Adventitious Root Formation in Relation to Changes in Compounds Involved in Rooting. Journal Plant Growth Regulation, **20**, 152-194 **(2001)**.

[37] D.Ríos, F.Avilés, M.Sánchez-Olate, R.Escobar, G.Pereira; Rooting rate variation related to subculture number diameter of chestnut Castanea sativa Mill.Microshoots. Agr.Técn., **65**, 258-264 **(2005)**.

[38] C.Flores, A.Cabañas, I.Peñalosa, R.Quintanar, J.Vásquez, M.Urzúa; EndógenousAuxin, AIA-oxidase and rooting in VignaradiataL.Wilczek induced by exógenous free and conjugated auxin. Revisión Fitotecnia, **32(1)**, 61-66 **(2009)**.

[39] A.Qaddoury, M.Amsa; Effect of exogenous indole butyric acid on root formation and peroxidase and indole-3-acetic acid oxidase activities and phenolic contents in dare palm off-shoots. Bot.Bull.Acad.Sm., **45**, 127-131 **(2004)**.

[40] P.J.Davies; The plant hormones their nature, occurrence and functions. In: Plant Hormones, Biosynthesis, Signal Transduction, Action. Kluwer Acad.Pub.Neitherlands, 5-6 **(2004)**.

[41] R.Benjamins, B.Scheres; Auxin, The looping star in plant development. Ann.Rev.Plant.Biot., **59**, 443-465 **(2008)**.

[42] B.Bartel, S.Le Clere, M.Magidin, B.K.Zolman; Inputs to the activeindole-3-acetic acid pool: de novo synthesis, conjugate hydrolysis, andindole-3-butyric acid b-oxidation. Journal of Plant Growth Regulation, **20**, 198–216 **(2001)**.

[43] N.Robert-Klever, J.Albreechlová, S.Fleig, N.Huck, W.Michalke, E.Wagner, V.Speth, W.Neuhrus, Ch.Fischer-Iglesias; Plasma Membrane H^+ -ATPase Is Involved in Auxin- Mediated Cell Elongation during Wheat Embryo Development. Plant Physiology, **131**, 1302-1312 **(2003)**.

[44] A.Walz, C.Seidel, G.Rusak, S.Park, J.Cohen, J.Ludwig-Muller; Heterologous expression of IAP1, a seed protein from bean modified by indole-3-acetic acid, in Arabidopsis thaliana and Medicagotruncatula. Planta, **227**, 1047-1061 **(2008)**.

[45] J.J.Campanella, S.M.Smith, D.S.Leibu, J.Wexler, Ludwig-Müller; The auxin conjugate hydrolase family of Medicagotruncatulaand their expression during the interaction with twosymbionts. J.Plant Growth.Reg., **27**, 26-38 **(2008)**.

[46] J.J.Campanella, S.Scott, J.Ludwig-Muller; Truncation of Medicago truncatula Auxin Conjugate Hydrolasas Alters Substrate Specificity. Plant Molecular Biology.Rep., **29**, 745-752 **(2011)**.

[47] J.Ludwig-Müller, E.Epstein, W.Hilgenberg; Auxin-conjugate hydrolysis in Chinese cabbage: characterization of an amido hydrolase and its role during infection with club root disease. Physiol.Plant, **97**, 627–634 **(1996)**.

[48] J.J.Campanella, V.Bakllamaja, T.Restieri, M.Vomacka, J.Herron, M.Pamerson, S.Shahtaheri; Isolation of an ILR1 auxin conjugate hydrolase homologue from Arabidopsissuecicum. Plant.Growth. Reg., **39**, 175-181 **(2003)**.

[49] J.J.Campanella, A.Olajide, V.Magnus, J.Ludwig-Miller; A novel auxin conjugate from wheat with

subsmate specificity for longer side-chain auxin amide conjugates. Plant.Physiol., **135**, 2230-2240 **(2004)**.

[50] B.Bartel; Endogenous IBA provides IAA for cell expansion. Plant Physiology Preview, **(2010)**.

[51] W.Teale, I.Paponov, K.Palme; Auxin in action: signalling, transport and the control of plant growth and development. Nature Review Molecular Cell Biology, **7**, 847–859 **(2006)**.

[52] L.Strader, A.Hendrickson, Jerry Cohen, B.Bartel; Conversion of Endogenous Indole-3-Butyric Acid to Indole-3-Acetic Acid Drives Cell Expansion in Arabodopsis Seelings. Plant Physiology, **153**, 1577-1586 **(2010)**.

[53] A.Muller, E.W.Weiler, Indolic constituents and indole-3-acetic acid biosynthesis in the wild-type and a tryptophan auxotroph mutant of Arabidopsis thaliana. Planta, **21**, 1855–863 **(2000)**.

[54] B.K.Zolman, B.Bartel; An Arabidopsis indole-3-butyric acid response mutant defective in PEROXIN6, an apparent ATPase implicated in peroxisomal function. Proceedings of the National Academy of Science of the USA., **101**, 1786–1791 **(2004)**.

[55] Li.Shi-Weng, Xue.Lingui, Xu.Shijian; Mediators, Genes and Signaling in Adventitious Rooting Huyuan Feng2 &Lizhe An2Bot.Rev., **75**, 230–247 **(2009)**.

[56] Ramirez-Carvajal, Gustavo, Davis, M.John; Cutting to the base Identifying regulators of adventitious rooting. Plant Signaling & Behavior Bioscience, **5(3)**, 281-283 **(2010)**.

[57] C.Kevers, J.F.Hausman, O.Faivre-Rampant, D.Evers, T.Gaspar; Hormonalcontrol of adventitious rooting: progress and questions. J.Appl.Bot., **71**, 71-79 **(1997)**.

[58] G.De Klerk, W.Krieken, J.De Jong; The formation of adventitious root: new concepts, new possibilities.In vitro cell development. Biología. Plant., **35**, 189-199 **(1999)**.

[59] E.Caboni, M.G.Tonelli, P.Lauri, P.Iacovacci, C.Kevers, C.Damiano, T.Gaspar; Biochemical aspects of almond microcuttings related to in Vitro rooting ability. Biología Plantarum, **19**, 91-97 **(1997)**.

[60] G.De Klerk, H.Guan, P.Huisman, S.Marinova; Effects of phenolic compounds on adventitious root formation and oxidative decarboxylation of applied indoleacetic acid in Malus 'Jork 9'. Plant.Growth Regul, **63**, 175–185 **(2011)**.

[61] M.Latsague, J.Lara; Fenoles solubles y su relación con la inhibición de la rizogénesis en estacas de Nothofaguspumilio (POEPP ET ENDL.) Krasser. Gayana.Bot., **60(2)**, 90-85 **(2003)**.

[62] M.E.Morris, S.Zhang; Flavonoid-drug interactions: Effects of flavonoids on ABC transporters. Life.Sci., **7**, 2116-2130 **(2006)**.

[63] D.Lewis, M.Ramirez, N.Miller, P.Vallabhaneni, K.Ray, R.Helm, L.B.Winke, G.Muday; Auxin and Ethylene Induce Flavonol Accumulation through Distintc Transcriptional Networks. Plant Physiology, **156**, 144-164 **(2011)**.

[64] G.K.Muday, A.Rahman; Auxin transport and the integration of gravitropic growth. In S Gilroy, P Masson, eds, Plant Tropisms. Blackwell Publishing, Oxford, UK, 47–48 **(2006)**.

[65] D.Santelia, S.Henrichs, V.Vincenzetti, M.Sauer, L.Bigler, M.Klein, A.Bailly, Y.Lee, J.Frimi, M.Geisler, E.Martonoia; Flavonoids Redirect PIN mediated Polar Auxin Fluxes during Root Gravitropic Responses. The Journal of Biological Chemistry, **263**, 31218-31226 **(2008)**.

[66] B.Kuhn, M.Geisler, L.Bigler, C.Ringli; Flavonols Accumulated Asymmetrically and Affect Auxin Transport in Arabidopsis. Plant Physiology, **156**, 585-595 **(2011)**.

[67] Foster M.Dolores; Flavonoides en citrus, distribución, modulación por fitorreguladores y posible función fisiológica. Departamento Biología Vegetal Programa de Doctorado Biología vegetal, **(1996)**.

[68] M.Geisler, O.Kotukisaoglu, K.Billion, J.Berger, B.Saal, R.Bouchard, N.Fragne, Z.Koncz-Katman, C.Koncz, R.Dudter, J.J.Blakeslee, A.S.Murphy; TWISTED DWARF1, a unique plasma membrane-anchored immune philin-like protein, interacts with Arabidopsis multidrug resistance-like transporters AtPGP1. Mol.Biol.Cell, **14**, 4238-4249 **(2003)**.

[69] K.Terasaka, J.J.Blakeslee, B.Titapiwantanakun, W.A.Peer, A.Bandyopadhyay, S.N.Makam, O.R.Lee, E.L.Richards, A.S.Murphy, F.Sato; PGP4, an ATP binding cassette p-glycoprotein, catalyzes auxin transport in Arabidopsis thaliana roots. Plant Cell, **17**, 2922-2939 **(2005)**.

[70] A.Alvarez De Felipe, M.Pulido; ABC Transporters: consequences of interaction with flavonoids.Boletín Latinoamericano y del Caribe de Plantas medicinales y aromáticas, **7(6)**, 298-311 **(2008)**.

[71] S.Zhang, M.E.Morris; Effects of the flavonoids biochanin A, morin, phloretin and ilymarin on Pglycoprotein-mediated transport. J.Exp.Pharmacol The, **304**, 1258-1267 **(2003)**.

[72] C.Buer, G.Muday, P.Sukumar; Ethylene modulates Flavonoids Accumulation and Gravitropic Responses in Roots of Arabidopsis. Plan Physiology, **140**, 1384-1396 **(2006)**.