African Journal of Biotechnology Vol. 10(9), pp. 1527-1523, 28 February, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB09.976
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Micropropagation of *Ocotea porosa* (Nees & Martius) Barroso

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Accepted 12 May, 2010

The objective of this work was the establishment of a micropropagation protocol for *Ocotea porosa* by multiplication of shoots from axillary buds. Different concentration of BAP (0; 2.5; 5 or 10 µM) or BAP+KIN (0; 1.25; 2.5 or 5 µM) were investigated to optimize the multiplication. Shoot growth was stimulated with reduced concentration of BAP (0; 0.5; 1 or 1.5 µM) or KIN (0.5 or 1µM) or activated charcoal (0.5; 1; 2 or 3 gl^{-1}). For root induction different concentrations of IBA (0; 1.25; 2.5; 5 or 10 µM) or (0; 2.5; 5 or 10 mM) were applied. The highest mean multiplication rate was observed in the fourth subculture with 5 µM BAP, reaching 5.3 shoots per explant. The shoots elongated in culture medium supplemented with 2 gl^{-1} activated charcoal and presented bigger leaves than on medium with reduced concentration of BAP. The shoots rooted on medium contains 10 µM IBA or after pulse treatment of 10 mM (68.7 and 62.6% of rooting, respectively). The survival rate of the plants was 56.7%. This study showed that *O. porosa* micropropagation is feasible; however it needs further research in order to increase plant survival.

Key words: 6-Benzylaminopurine, multiplication, rooting, *in vitro* culture, apical shoots, native specie.

INTRODUCTION

Ocotea porosa (Nees & Martius) Barroso (Lauraceae), is a tree native to the Mixed Ombrophyllous forest (Araucaria Forest) of Brazil. Its wood is of excellent quality and widely appreciated. It is exported for the confection of luxury furniture and as timber for constructions and carpentry (Carvalho, 2003). *O. porosa* is an endangered specie, given its exploration for wood and the deforestation due to agriculture expansion in its natural occurrence area (Carvalho, 2003; Caldato et al., 1999; Neto et al., 2002).

The seeds are recalcitrant, it has a strong tegument dormancy and their germination is irregular, making its natural propagation difficult (Carvalho, 2003). Moreover the rooting rate of cuttings is low (Inoue and Putton, 2007), limiting its vegetative propagation for replanting.

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Abbreviations: BAP, 6-Benzylaminopurine; **KIN,** kinetin; **IBA,** indol-3-butyric acid.

Micropropagation constitutes a powerful tool for *ex situ* conservation programs of the rich flora, especially for species with reduced populations or low production of seeds (Debnath, 2004), or for difficult to regenerate species when in natural conditions, as it is the case for *O. porosa.* Considering these difficulties, micropropagation represents an important tool for its conservation. This technique may be successfully used for mass propagation of selected genotypes, conservation and genetic improvement (Thorpe and Kumar, 1993). Moreover, it facilitates the rapid establishment of a large number of plants, with a minimum impact on the endangered wild populations (Debnath, 2004).

The production of plants from axiliary buds or shoots has proved to be the most generally applicable and reliable method of true - to - type *in vitro* propagation (George et al., 2008). There are few studies about micropropagation of species from Lauraceae family and none for *O. porosa*. However, for other forest species of the Lauraceae family, studies have already been carried out to establish protocols of regeneration by somatic embryogenesis, such as *Laurus nobilis* (Canhoto et al., 1999),

Persea americana Mill (Witjaksono et al., 1999), and *Ocotea catharinensis* (Moura-Costa et al., 1993; Viana, 1998; Viana and Mantell, 1999; Santa-Catarina et al., 2003, 2004; Moser et al., 2004; Santa-Catarina et al., 2005), because of the economical and ecological importance of *O. porosa,* this study aimed to establish a micropropagation protocol for plant mass production.

MATERIALS AND METHODS

Plant material. Seeds of O. porosa were collected from a natural forest at Colombo-Parana, Brazil, in 2006. The pulp of the mature fruits was removed manually and the seeds washed in current water. The seeds were then sowed in trays containing sieved soil and Plantmax® substrate (3:1). The trays were kept in a greenhouse with controlled temperature (18–23ºC). After germination the seedlings were transplanted into plastic bags (15x25 cm) containing the same substrate. Manual irrigation was made directly in the substrate. The one-year-old plants were trimmed and shoots tip were collected and used as explants.

In vitro establishment. shoots tip (approximately 2 cm in length), containing one or two axillary buds, were surface-sterilized in 70% (v/v) ethanol for 30 s, followed by immersion for 10 min in a 0.25 and 0.5% (v/v) sodium hypochlorite solution plus 0.1% Tween20® and finally rinsed six times in sterile distilled water. They were placed in flasks containing a medium composed of half-strength MS salts (Murashige and Skoog 1962), full MS vitamins, sucrose (30 gl^{-1}), polyvinylpolypirrolidone (0.5 gl^{-1}) and Micromed® agar (6 gl^{-1}). The flasks used for establishment in vitro and multiplication were 3 cm in diameter and 7 cm in height with 15 ml medium each and covered with aluminum foil. Each treatment consisted of ten flasks repeated four times, with one shoot tip per flask (n=40). The percentage survival of the shoot tips was evaluated after four weeks.

Multiplication. The shoot tips were cultured in a MS culture medium supplemented with 6-Benzylaminopurine (BAP) (0, 2.5, 5 or 10 µM) or with combinations of BAP and Kinetin (KIN) (0+0, 1.25+1.25, 2.5+2.5 or 5+5 µM), sucrose (30 gl^{-1}) and solidified with BBL® agar (6 gl^{-1}). Each treatment consisted of eleven flasks repeated four times, with one shoot per flask (n=44). The recording of the average number of shoots per explant and the mean length of axillary shoots was done after four weeks during four subcultures. The new axillary shoots were individualized and subcultured every four weeks. The mean multiplication rate was calculated by counting the axillary shoots originated in each subculture.

Elongation. The effect of reduced concentrations of BAP or KIN and the effect of the activated charcoal on the elongation of the shoots were studied. Shoots tips (0.5 to 1.5 cm long) originated from the fourth subculture of the multiplication phase were used. The percentage of elongation of the shoots was evaluated after six weeks.

In the first experiment shoots tip were transferred onto MS culture medium supplemented with BAP (0; 0.5; 1 or 1.5 µM) or KIN (0.5 or 1 µM), sucrose (30 gl-1) and BBL® agar (6 gl-1). Each treatment consisted of four replicates of two flasks each and five shoots per flask (n= 40).

In the second experiment shoots tip were placed onto MS culture medium containing activated charcoal (0.5, 1, 2 or 3 gl-1), sucrose (30 gl-1) and BBL® agar (5.5 gl-1). Each treatment consisted of four replicates of four flasks each and five shoots per flask (n= 80). In order to calculate the percentage of shoot elongation the apical length was multiplied by 100 and divided by the initial length.

Root induction. Shoots tip (approximately 2 cm long) grown on the elongation medium with activated charcoal had their base cut in double bevel and two apical leaves were maintained. They were placed onto a half-strength MS medium supplemented with indol-3-butyric acid (IBA) (0, 1.25, 2.5, 5 or 10 µM), sucrose (30 gl-1) and solidified with BBL® agar (5.5 gl-1). The cultures were maintained in the dark for 7 days and then transferred onto the same medium without IBA. The experimental design was completely randomized with four replicates of two flasks each and five shoots per flask (n=40). After four weeks, the percentage of rooted shoots, the mean number of roots per shoot tip and mean length of roots were calculated.

Another treatment consisted of the immersion of the shoot base into IBA solutions (0, 2.5, 5 or 10 mM) (dissolved in NaOH 0.1N) for 10 min. The shoots tip were placed onto a half-strength Woody Plant Medium (Lloyd and McCown 1980) with 20 gl-1 sucrose and BBL® agar (5.5 gl^{-1}). Four replicates of two flasks were done in each treatment, with five shoots per flask (n=40). After four weeks, the percentage of rooted shoots, the mean number of roots per shoot and mean length of roots were calculated.

Culture conditions. The cultures were incubated at day/night temperatures of 25±2°C/18±2°C, under white fluorescent light (40 µmol m-2 s-1) and a 16 h photoperiod. Experimental design was completely randomized. The flasks used during initiation and multiplication had 3 cm in diameter and 7 cm in height, contained 15 ml of medium each and were covered with aluminum foil. During elongation and root induction flasks of 7 cm in diameter and 15 cm height were used, with 40 ml of medium in each and closed with polypropylene caps. All media had their pH adjusted to 5.8 with NaOH 0.1 N or HCl 0.1 N and were autoclaved for 20 min at 121°C.

Transplant and acclimatization. The rooted plants were pre-acclimatized in the growth room for 48 h and the flasks were gradually opened. They were subsequently removed from culture vessels and planted into plastic tubes (53 cm3) containing the following mixtures: Plantmax® HT substrate (100%); Plantmax® HT + charcoal (3:1); Plantmax® HT + carbonized rice hull (2:1) and Plantmax® HT + carbonized rice hull + sieved earth (2:1:1). They were maintained under intermittent mist (1 min every 5 min) in a greenhouse with a temperature of 24±2ºC and RH of 90±2%. The percentage of plant survival was recorded after 4 weeks.

Statistical analyses. The data were submitted to an analysis of variance (ANOVA) and Tukey´s test at $P \leq 0.05$ was applied, using the MSTATC program.

RESULTS AND DISCUSSION

In vitro establishment

The shoots tip disinfestation with 70% (v/v) ethanol for 30 s followed by immersion for 10 min in a 0.25 or 0.5% (v/v) sodium hypochlorite solution was efficient. The explants presented a high percentage of survival (95) and 5% of fungal contamination.

Another factor that probably contributed to have a low percentage of contamination was the fact that the irrigation was applied directly in the substrate and that the mother plants were maintained in a greenhouse. The disinfestation of explants collected in the field is more difficult, therefore the contamination is frequently high when compared with explants collected from young plants kept in a greenhouse. The establishment of aseptic cultures is one of the advantages of the micropropagation technique for the regeneration of plants.

Differently from the results described in this paper, Wang et al. (1991) obtained only 5.1% of the shoots of

Table 1. The influence of three BAP concentrations on axillary shoot formation from shoot tips of *Ocotea porosa* after four serial subcultures.

BAP (µM)	Subculture cycles							
	Subculture 1		Subculture 2		Subculture 3		Subculture 4	
	Mean multiplication rate	Mean length shoots	Mean multiplication rate	Mean length shoots	Mean multiplication rate	Mean length shoots	Mean multiplication rate	Mean length shoots
0	1.6^{b}	1.0^{a}	1.9^{c}	0.9^{ab}	1.7^{b}	0.4^{b}	1.7^{b}	0.5^{a}
2.5	2.5^{b}	0.9^{a}	2.9^{bc}	1.1^{a}	3.0^{b}	1.0^{a}	3.1^{b}	1.2^{a}
5	3.8^{a}	1.0^{a}	3.9^{ab}	0.9^{ab}	4.8^{a}	0.8^{ab}	5.3^{a}	0.8^{a}
10	3.9^{a}	0.9^{a}	4.3^{a}	0.7^{b}	3.3^{ab}	0.8^{ab}	3.2^{b}	0.7^{a}

Mean values followed by the same letter are not significantly different at P<0.05 according to Tukey´s test.

Figure 1. Micropropagation of *O. porosa.* (A) Shoots tip in a MS culture medium supplemented with 10 µM BAP during the 3d subculture. (B) Shoots tip rooted in half-strength WPM medium supplemented with 10 mM IBA after 4 weeks. (C) Plant acclimatized under greenhouse conditions with new leaves, 4 w after transplant (bar = 2 cm).

Sassafras randainense (Lauraceae) disinfested using 75% ethanol followed by 0.5% sodium hypochlorite solution (5 min).

Multiplication. during the first and second subcultures on BAP media, the mean multiplication rate increased proportionally to the rise in BAP concentration (Table 1) whereas in the third and fourth subcultures it increased up to a BAP concentration of 5 µM and then decreased at 10 µM (Table 1 and Figure 1a) reaching 5.3 shoots per explant in the fourth subculture (5 µM BAP) (Table 1).

BAP was effective to remove the apical dominance of shoots. The mean multiplication rate increased with the number of subcultures and increase of BAP concentration, reaching 5.3 shoots per explant after the fourth subculture on medium containing 5 µM BAP. The addition of BAP to the culture medium was essential for *O. porosa* multiplication, as it was also observed for other species such as *S. drummondii,* for which the increase of shoot number per plant paralleled the increasing BAP concentrations in the culture medium (Cheepala et al., 2004). The number of axillary shoots of *Quercus semecarpifolia* formed per explant increased with increasing BAP concentration (Tamta et al., 2008).

In this experiment, during the third subculture the highest BAP concentration tested (10 µM) induced shoot necrosis (21.2%), thus reducing the mean multiplication rate in this subculture and in the fourth one. The mean length of the axillary shoots decreased with the increase of the BAP concentrations and this was also observed for other species as *Cercis canadensis* (Mackay et al., 1995). According to George et al. (2008), elevated levels of cytokinin may cause many small shoots to be produced, which typically fail to elongate. Tamta et al. (2008) reported that while shoot multiplication was found to increase after successive subcultures there was a concomitant decrease in shoot height, but it could be restored by a single culture on medium with BAP reduced to 1 µM.

Concerning the mean length of *in vitro* regenerated axillary shoots, there was little variation between the BAP treatments tested. In the third and fourth subcultures the mean length of the axillary shoots was higher with the increase of the BAP concentrations up to 5 µM, while at10 µM BAP it presented a reduction (Table 1). In the first and second subcultures the rise of BAP concentration (10 µM) reduced the mean length of the

Table 2. Influence of different BAP and KIN concentrations on axillary shoot formation from shoot tips of *Ocotea porosa* during four serial subcultures.

BAP+KIN (µM)	Subculture Cycles							
	Subculture 1		Subculture 2		Subculture 3		Subculture 4	
	Mean multiplication rate	Mean length shoots	Mean multiplication rate	Mean length shoots	Mean multiplication rate	Mean length shoots	Mean multiplication rate	Mean length shoots
0	1.8^{b}	0.7^{a}	1.4^{b}	0.5^{a}	1.4^{b}	0.6^{a}	1.5^{b}	0.3^{a}
1.25+1.25	2.7^{a}	1.0^{a}	2.4^{ab}	0.7^{a}	2.3^{ab}	0.5^{a}	2.1^{ab}	0.4^{a}
2.5+2.5	2.5^{a}	0.9^{a}	2.7^{a}	0.9^{a}	3.0^{a}	0.7^{a}	3.6^{a}	0.7^{a}
5+5	2.9^{a}	0.9^{a}	3.0^{a}	0.7^{a}	3.4^{a}	0.7a	3.5^{a}	0.7^{a}

Mean values with the same letters within columns are not significantly different at P<0.05 according to Tukey´s test.

Table 3. Elongation of *O. porosa* shoots on MS medium supplemented with BAP or KIN.

Elongation (%)	BAP (µM)				Kin (µM)	
	0	0.5	1	1.5	0.5	1
	47.9^{a}	59.7^{a}	79.3^{a}	61.8^{a}	25.9^{a}	42.8^{a}

Evaluated 6 weeks after culture. Mean values followed by the same letter are not significantly different at P<0.05.according to Tukey´s test.

axillary shoots (Table 1).

In the experiment of multiple shoots induction on media containing combinations of BAP and KIN, in the first subculture the mean multiplication rate increased with the cytokinin concentrations (Table 2). An increase was also observed in the mean multiplication rate of the following subcultures (Table 2). The highest mean multiplication rate was 3.6 shoots per explant (Table 2).

The combination of BAP and KIN was not efficient to induce elongation of shoots during the multiplication phase, though the literature indicates the promotion of elongation induced by kinetin in some species (Mackay et al., 1995; Pattnaik et al., 1996). In this experiment the use of isolated or combined cytokinins in the culture medium induced the loss of apical dominance and favored the development of *O. porosa* axillary shoots. The mean multiplication rate was 1.8 in the cytokinin-free medium, while it was 3.6 in the media containing both cytokinins. However, when BAP was tested alone, the mean multiplication rate was higher (5.3 shoots per explant) than when BAP and kinetin were combined (3.6 shoots per explant).

The mean length of the axillary shoots had little variation in function of cytokinin concentrations. In the first and third subcultures, the mean length of the axillary shoots varied very little (Table 2) whereas, in the fourth subculture, a increase of the mean length with the increase of the BAP and KIN concentrations was observed (Table 2).

Elongation

Though the differences between the results obtained with low concentrations of BAP and KIN were not significant, the treatment with 1 µM BAP resulted in an increase of 79.3% in shoot elongation (Table 3). When activated charcoal was added to the culture medium, this increase was proportional to the rise in activated charcoal concentration up to 2 gl^{-1}, but the elongation tendency was reduced in the medium containing 3 gl^{-1}. Moreover the shoots cultured in the medium containing 2 gl^{-1} had leaves more developed than in the other treatments (Table 4).

In the presence of a low concentration of BAP (1 µM) an increase in the elongation of the shoots was observed, as well as in the culture medium containing activated charcoal, but the last medium was less efficient than the first one. Shoot quality can be improved by reducing the level of cytokinin in subcultures immediately before shoots are harvested for rooting and by using a different cytokinin or a mixture of them (George et al., 2008). On the other side, shoots elongated in the presence of activated charcoal presented more expanded leaves, which can favor the rooting, and the activated charcoal is also known to adsorb cytokinins and other compounds. Activated charcoal has been frequently added into the culture medium for plant tissue with success (Van Winkle et al., 2003). It can improve or regulate plant growth *in vitro* absorbing compounds secreted from cultured

Table 4. Elongation of *Ocotea porosa* shoots on MS medium supplemented with activated charcoal.

Elongation (%)	Activated charcoal (g L^{-1})			
	0.5	1	2	3
	25.3^{b}	30.9^{ab}	$41,4^{a}$	27.9^{ab}

Evaluation after 6 weeks of culture. Mean values with the same letters are not significantly different at P<0.05 according to Tukey´s test.

Table 5. The influence of different IBA concentrations on rooting rate, mean number of roots, mean length of the roots of *O. porosa* microcuttings after 4 w.

AIB (µM)	Rooting rate (%)	Mean number of roots	Mean length of the roots (cm)
0	35.0^{a}	1.2^{a}	0.3^{c}
1.25	42.5^{a}	1.1^{a}	0.4^{bc}
2.5	45.0^{a}	1.7^{a}	0.5^{abc}
5	65.0^{a}	1.9^{a}	0.9^{a}
10	68.7^{a}	1.6^{a}	0.8^{ab}

Mean values with the same letters within columns are not significantly different at P<0.05.according to Tukey´s test.

tissues or present in agar that would otherwise inhibit growth (George et al., 2008). Consequently, the use of culture medium supplemented with 2 gl^{-1} activated charcoal for *O. porosa* shoots elongation can be interesting as it will reduce the costs of the micropropagation process in this species.

Root induction

In order to obtain a better rooting rate, rhizogenesis was induced in a medium supplemented with auxin for seven days in the dark, followed by transfer into an auxin-free medium for root growth. It was observed that the increases in rooting rate and mean length of roots were proportional to the rise in IBA concentration. Rooting rate reached 68.7% and the mean length was 0.8 cm in the medium containing 10 µM IBA (Table 5). The mean number of roots increased up to a concentration of 5 µM IBA (1.9 roots per explant) while at 10 µM IBA a reduction was observed (Table 5).

Rooting of *O. porosa* microcuttings was first tested in half-strength MS medium containing or not containing IBA (1.25; 2.5 or 5 µM) during four weeks. The maximum rooting rate in this case was 30% in the presence of 2.5 µM IBA (data not shown). The results showed that shoots cultivated in medium supplemented with auxin during four weeks were poorly rooted and that it was necessary to induce rooting in culture medium containing auxin and then transfer the plants to an auxin-free medium. The percentage of rooted shoots was 68.7%, different from the results obtained with rooting in culture medium with auxin during four weeks (30%).

The 10 min pulse-treatment applied to the microcuttings raised the rooting percentage which was proportional to the rise in IBA concentration, getting 62.6% of shoots rooted (10 mM IBA) (Table 6 and Figure 1b). The rooting was observed in all the treatments, however without IBA application, the rooting rate was low (15.3%), indicating the need of auxin treatment to induce rhizogenesis in this specie. The highest IBA concentration induced a reduction in the mean length of the roots. The pulse-treatment of 10 mM induced an average of 2 roots per shoot (Table 6).

Root induction by pulse treatment presented results similar to those obtained with root induction during 7 days. The pulse treatment is thus an alternative to optimize the rooting of this specie and it will also allow to reduce the costs of culture medium during this stage.

However, a longer time (15 min) of shoot immersion into auxin solution or higher concentrations need to be tested to increase the percentage of rooting and root number. *O. porosa* microcuttings had a low rooting rate when cultured for 7 days in an auxin-free culture medium (35%) and after pulse-treatment (15.3%), indicating that this specie may be considered a difficult - to - root forest species, according to the classification of Marks and Simpson (2000). Vieitez et al., (2007) recommended pulse-treatment (1- 2 min) with IBA (4.9 µM) for induction of roots in *Castanea sativa*.

Transplanting and acclimatization

The plants transplanted in the greenhouse did not present any morphological modification during the first two weeks. However, the pre-acclimatization in the growth room was not suitable to give a good survival rate, as the maximum survival rate was 56.7% after four weeks for plants grown in Plantmax® substrate and carbonized rice

Table 6. The influence of pulse-treatment with different IBA concentrations on rooting rate, mean number of roots and mean length of the roots of *O. porosa* after 4 w.

IBA (mM)	Rooting rate (%)	Mean number of roots	Mean length of the roots (cm)
0	15.3[b]	1.3[a]	0.5[a]
2.5	35.0[ab]	1.9 [a]	0.7[a]
5	61.1[a]	1.9 [a]	0.6[a]
10	62.6[a]	2.0 [a]	0.5[a]

Mean values with the same letters within vertical colunns are not significantly different at P<0.05.according to Tukey´s test.

Table 7. Percentage of survival of *O. porosa* plants cultured in several substrates under greenhouse conditions, after 4 w.

Substrate type	Number of plants	Survival (%)
Plantmax®[*]	24	25.0
Plantmax® + charcoal (3:1)[*]	24	41.7
Plantmax® + carbonized rice hull (2:1)[**]	30	56.7
Plantmax® + carbonized rice hull + sieved earth (2:1:1)[**]	30	53.3

[*]Plants cultured in culture medium without activated charcoal.
[**]Plants cultured on a half-strength MS culture medium supplemented with 1 gl^{-1} activated charcoal.

hull (2:1) (Table 7 and Figure 1c).

After four weeks the percentage of survival was 56.7%. One of the reasons for high plant mortality may be due to liberation of phenolic compounds during the acclimatization, which caused plant oxidation. Another factor that may have contributed negatively to plant survival in the greenhouse is the time the rooted cuttings were maintained in the culture medium; if this period is too long, the roots may turn senescent and less functional. Though the plant growth was not evaluated during the acclimatization, it was slow during the first step of the culture. It is therefore necessary to consider the conditions of transplanting as well as the functional state of the *in vitro* newly formed roots. A similar result (60%) was described for acclimatization of *Sesbania drummondii,* with reduction of this percentage after three months (30%) (Cheepala et al., 2004).

Conclusion

In the present study, a protocol of *O. porosa* micropropagation was described. The presence of BAP in the culture medium was important for the development of shoots and an average of 5.3 shoots per explant was obtained with 5 µM BAP after the fourth subculture. Shoots elongated on a culture medium containing 2 gl^{-1} activated charcoal. The root formation can be obtained with pulse - treatment of 10 mM IBA. For acclimatization, further studies must be carried out during this stage, such as phytochemical analyses and anatomical studies, in order to increase the plant survival.

ACKNOWLEDGEMENTS

Luciana Luiza Pelegrini thanks CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for the M. Sc grant and Marguerite Quoirin for manuscript revision.

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