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Micropropagation of *Isoplexis chalcantha* Svent, O´Shanahan from Mature Plants

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Abstract

Culture medium requirements for micropropagation of *Isoplexis chalcantha* was achieved for the first time after high degree of contamination and phenolic exudates were detected and solved. Cultures were established from axillary shoots using juvenile branches collected from this medicinal plant. Most satisfying results were obtained using a solidified and a modified MS medium (NO_3^- : NH_4^+ ratios) enriched with ascorbic acid or soluble PVP plus GA_3, BAP and NAA. Explants (nodal segments) were used for *in vitro* shoots multiplication and best results were achieved with modified MS plus BAP and auxins. Vigorous shoots rooted without symptoms in the half-strength modified MS enriched with low concentration of IBA.

Introduction

Isoplexis chalcantha (Scrophulariaceae) is a very important medicinal and ornamental endemic species to the Canary Islands, Spain. Seed germination of *I. chalcantha* and seedling survivality were low (unpublished data). On the other hand, tissue culture technology provides an alternative method for solving these problems. Reports on *in vitro* culture of *Isoplexis* species are limited (Mederos-Molina 1995, Schaller and Kreis 1996, Arrebola et al. 1997). The purpose of this study is to report for the first time, a micropropagation method of this interesting species and may be economically exploited as medicinal and ornamental plants (Varela and Santos 2002).

Materials and Methods

Micopropagation of *Isoplexis chalcantha* from mature plants has been carried out by axillary shoots of seven-month-old collected from 20 years old mature plants. Explants were washed with 1 and 2% benomyl solutions for 10 and 15 min, respectively. The cuttings were then shaken in 96% (v/v) ethanol for 5 min. Next,

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they were placed for 15 to 25 min in 3 and 5 % (v/v) sodium hypochlorite or in 0.1, 0.15, 0.2 and 0.25% (v/v) $HgCl_2$ solutions with 1 ml of Tween 20 surfactant for 5, 7 and 10 min. Finally, the axillary shoots were washed three times with sterile distilled water. To investigate the effect of antioxidants on browning exudate, ascorbic acid and soluble polyvinyl pirrolidone (PVP), (0.5, 1, 1.5 and 2 mM, respectively) were used. After dissection single axillary shoots were placed into recipient containing 15 ml culture medium.

For *in vitro* establishment of shoots normal MS was tested. Control treatment including the original MS salt formulation (40 NO_3^- : 20 NH_4^+). Again, this culture medium was employed with various modifications involving the addition of different concentrations of KNO_3 and NH_4NO_3 (NO_3^- : NH_4^+), and supplemented with PVP + BAP combined with NAA (Table 1). All macroelement media were supplemented with Fe-EDTA and MS microelement plus 2.96 μM thiamine-HCl, 2.96 μM pyridoxine-HCl, 0.56 μM myo-inositol, 202 mM glucose and 0.5% (w/v) agar (Difco Bactor agar). On the other hand, culture media were supplemented with GA_3 + BAP alone or combined with IAA and NAA (Table 2). For *in vitro* rooting, the shoots were cultured in a normal and modified MS media containing 0.5 mM soluble PVP plus 1.11 μM BAP and low concentrations of NAA or IBA (Table 3).

In all the culture media the pH was adjusted to 5.6 which was sterilized under 0.5 atmospher at 115ºC for 15 min. Cultures were incubated in a growth chamber at 25 ± 1ºC under 16 h photoperiod regimes and provided by Sylvania Gro-Lux fluorescent lights [(60 μmol/m^2/s), (Tables 1 and 2)], [(30 μmol/m^2/s), (Table 3)] and 77 ± 2.5% relative humidity. Each treatment had 24 replicates and all experiments were conducted at least twice.

Following parameters such as explants with developed shoot (ExDS); shoot and root qualities (3 = vigour stems, 2 = slightly vigour stems and 1 = not vigour stems) and elongation of these was evaluated to study the organogenesis ability of explants concerning all the above mentioned culture media. Data were subjected to analysis of variance, and mean comparison was determined by Tukey-Kramer HSD at $p = 0.05$ of probability.

Results and Discussion

High contamination rates (80%) were often observed during the preliminary *in vitro* experiments of *Isoplexis chalcantha*. Results indicated that pre-soaking of the explants with 1% benomyl (15 min) is a prerequisite to avoid fungal contamination detrimental to reduce vitality of the shoots growth. Sodium hypochlorite is one of the most widely used chemical compounds for surface sterilization explants of ornamental plants (Hermann 2007). However, there was no significant effect on decontamination in *I. chalcantha*. Thus, elimination of

contamination was controlled by washing the explants with 0.15% (v/v) $HgCl_2$ solutions plus 1 ml of Tween 20 surfactant for seven min.

During the preliminary experiments of *I. chalcantha* the culture media show polyphenolic exudates with inhibition of meristem division and necrosis (data not shown). The prevention of browning phenomenon is essential to avoid necrosis during the establishment of this tissue in culture. Ascorbic acid (1.5 and 2.0 mM) or soluble PVP removed phenolic substances but they were found to be injurous for the explants (data not shown). A fairly rapid browning of the explants was very common in woody medicinal species (Hermann 2007). *I. chalcantha* tissue produces phenolic compounds which are easily oxidized but results in the death of explants. However, ascorbic acid and soluble PVP not always stop the blackening of tissue culture explants (Mederos and Trujillo 1999, Hermann 2008). On the other hand, during the preliminary experiments of this medicinal species, BAP was far more effective than Kin for inducing growth and development of shoots (data not shown). The same result was achieved before with explants collected from seedlings of *I. canariensis* and cultured in solid and liquid MS media (Mederos-Molina 1995). Schaller and Kreis (1996) obtained quite different results, because micropropagation of juevenile explants collected from seedling of *I. canariensis* was effective in liquid basal MS supplemented with Kn. Different response which was observed with the same species may be due to the origin of this plant material, the environmental conditions and also, the age of explants (shoots) used for the *in vitro* cultures were different. Moreover, there was statistical difference in regeneration of shoots (Mederos-Molina 1995, Arrebola et al. 1997, Schaller and Kreis 1996). Among the five different culture media used, MS.3 (NO_3^- : NH_4^+ = 30 : 20 mM) and MS.4 (NO_3^- : NH_4^+ = 30 : 10 mM) were found to be better for *in vitro* establishment of shoots. Moreover, best results were obtained on the MS.3 and MS.4 culture media (Table 1) where the ExDS were 71 and 67%, respectively. The normal and modified MS media showed significant difference in terms of shoot length and shoot quality. The longest shoots developed in MS.3 (37 mm) and MS.4 (35 mm). Shoots obtained from MS.3 and MS.4 were longest but they were more vigorous and green in colour in MS.3. It was observed that in MS normal medium shoots had malformed leaves (data not shown). Further, only a few qualities of shoots (slightly vigour stems) were obtained in normal and modified MS.1, MS.2 and MS.5. It was also seen that the complete elimination of NH_4NO_3 from the MS stoped the growth and development of shoots (data not shown). Stimulatory effects of NH_4NO_3 were seemed to be related to the nitrogen metabolism which affected organogenesis. In the majority of media formulations commonly used for micropropagation of woody plant, the principal nitrogen sources are NO_3^- and NH_4^+. However, their relative concentrations vary widely and their effects on culture growth, biomass and yield were equally same (Hermann 2006, 2007,

2008). Furthermore, in this case the changes in the concentration of nitrate and ammonium limited the percentage of ExDS, shoot quality and elongation (Table 1). The macroelement solution proved to be a vital factor in the *in vitro* organogenesis of *I. chalcantha* as shown by the fact that during the *in vitro* establishment of different types of explant, significant differences ($p < 0.05$) have been observed on similar culture media (unpublished data), and similar behaviour was found in other species endemic to the Canary Islands (Mederos et al 2002a,b; 2006). The effect of low concentrations of KNO_3 and NH_4NO_3 was clearly seen for the stimulation of shoot emergence and further growth which is characterized by an increase in the length and quality of shoots. These observations conform the results of other medicinal woody species (Mederos-Molina 2002a,b; 2004a, 2006, 2007). More shoots proliferated under low concentration of ammonium nitrate (data not shown).

Table 1. ***In vitro*** **establishment of shoots of** ***Isoplexis chalcantha*** **on MS with 0.5 mM PVP and 1.11 µM BAP, 0.80 µM NAA, 202 mM glucose and 0.5 % difco bacto agar. Data were collected at 40 days of culture.**

Macroelement solutions[1]	NO_3^- : NH_4^+ (mM)	Percentage ExDS	Length of [2] shoot (cm)	Quality of shoots[2]
MS	40:20	42	1.0^{a} (0.3)	1.0^{a}(0.2)
MS.1	40:10	54	2.0^{c} (0.1)	2.0^{c}(0.3)
MS.2	30:30	62	2.9^{d} (0.2)	$2.0^{d,c}$(0.4)
MS.3	30:20	71	3.7^{f} (0.2)	3.0^{f}(0.2)
MS.4	30:10	67	$3.5^{e,f}$(0.2)	0.5^{e}(0.3)
MS.5	30:5	62	$1.3^{b,a}$(0.3)	1.5^{b}(0.2)

[1]NO_3^- : NH_4^+ : 2 : 1 (MS), (control); 4 : 1 (MS.1); 1 : 1 (MS.2); 1.5 : 1 (MS.3); 3 : 1 (MS.4); 6 : 1 (MS.5). Results were obtained from 33 days-old shoots. There were 24 whole shoots for each treatment. [2]Each value represents the mean of developed shoots ± S.E. Means in columns followed by the same letter are not significantly different at 0.05 level of confidence (Tukey-Kramer HSD´s test).

The above mentioned result showed the importance for testing a range of NO_3^- : NH_4^+ concentrations (mM) in an attempt to optimize the shoot proliferation. Moreover, the effect of plant growth regulators on growth, development and shoot proliferation was also observed. It was noted that of the various auxins tested for the initiation of axillary buds, IAA was found to be most effective. The highest percentage of developed shoots was achieved with combination of 1.44 µM GA_3, 2.22 µM BAP and 0.57 or 1.14 µM IAA (Table 2). The maximum response occurred with 0.57 µM IAA and 2.22 µM BAP combined with IAA (0.57 µM) had a significant effect on the per cent ExDS, shoot proliferation and length of shoots (Table 2). The low concentration of auxin was necessary for shoot proliferation of this medicinal and ornamental plants and these results are in agreement with other results previously reported (Mederos-Molina 2004a,b;

2006, 2007). It has been shown that the form and concentration of nitrogen sources have important influences on endogenous cytokinin synthesis (Mercier et al. 1997).

Table 2. Effect of various concentrations of BAP, IAA and NAA supplemented into the MS.3 (NO_3 : NH_4^+ = 30 : 20 mM) culture medium plus 1.44 GA_3 on shoot proliferation and length of shoots of *Isoplexis chalcantha*. Data were collected at 40 days of culture.

BAP	IAA (μM)	NAA	Percentage ExDS (%)	Shoot proliferation[(1)]	Length of shoots (mm) [(1)]
0	0	0	17	1.3 [a] (0.3)	10.3 [a] (0.3)
2.22	0.57	0	80	3.9 [e,d] (0.5)	41.5 [e] (0.7)
2.22	1.14	0	75	3.5 [d] (0.3)	30.2 [d] (0.6)
2.22	0	0.80	67	2.2 [b] (0.3)	22.1 [c,b] (0.4)
2.22	0	1.61	67	2.4 [c,b] (0.5)	19.0 [b] (0.5)

There were 24 whole shoots for each treatment. Percentage of whole shoots showing proliferation. [(1)]Each value represents the mean of developed shoots per culture ± S.E. Means in columns followed by the same letter are not significantly different at 0.05 level of confidence (Tukey-Kramer HSD´s test).

One of the difficulties to overcome initiating the rooting of *I. chalcantha* is brown exudation. This caused darkening of the shoots and led the shoots material to death. This problem has been previously solved in other woody plant species by some tissue cultures techniques (Mederos and Trujillo 1999, Mederos-Molina 2003). Further, the absence of browning in basal or modified media was observed in presence of 0.5 mg/l soluble PVP and low intensity light (17 ± 2.0 μmol/m^2/s) during first nine days of multiplication and rooting stages. There was no visible root development until two weeks after transfer to rooting media and

Table 3. Effect of various auxins at different concentrations in MS.3 (1.11 mg/l BAP plus 0.5 mg/l PVP) on root proliferation of *Isoplexis chalcantha*. Data were collected at 43 days of culture.

NAA (mg/l)	IBA	% of shoots with roots	No. of roots[(1)] per shoot	Length of[(1)] roots (mm)	Quality of roots [(1),(2)]
0	0	17	1.0[a] (0.3)	11[a] (0.5)	0.0
1.61	0	62	3.1[d,e,c] (0.7)	34[d,c] (0.4)	2.5
2.41	0	58	3.3[e,c] (0.6)	39[e] (0.6)	3.0
0	1.48	75	2.4[c,b] (0.4)	30[c,b] (0.5)	2.5 (17)
0	2.22	67	1.9[b] (0.3)	27[b] (0.4)	2.0 (19)

There were 24 shoots for each treatment. [(1)] Each value represents the mean of developed roots ± S.E. Means in columns followed by the same letter are not significantly different at 0.05 level of confidence (Tukey-Kramer HSD´s test). [(2)] (%) of plants with necrosis symptom.

percentage of rooting was clearly different ($p = 0.05$) on different auxin treatments (Table 3). Moreover, callus proliferated during the second week due to high doses of auxins [3.42 - 11.42 μM IAA; 3.22 - 10.74 μM NAA; 2.95 - 9.80 μM IBA], (data not shown in Table 3). Low concentration of 1.11 μM BAP combined with IBA produced more than 65% of rooted shoots and were significantly higher than NAA treatments ($p < 0.05$), (Table 3). However, the IBA and NAA treatments retarded the growth of roots (Mederos-Molina 2002a, 2003, 2004; Hermann 2006, 2008). On the other hand previous reports on *I. canariensis* mentioned the use of liquid culture media for micropropagation (Schaller and Kreis 1996; Arrebola et al. 1997), however in the same conditions hyperhydricity symptom was detected from *I. canariensis* and *I. chalcantha* and more experiments are in progress (Mederos et al. unpublished data). Finaly, this report provides a basis for further research on clonal propagation of *Isoplexis* species endemic to the Canary Island through tissue culture because, these medicinal species are a source of pharmacologically very interesting compounds.

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