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BRIEF COMMUNICATION

Micropropagation of *Ailanthus altissima* and *in vitro* heavy metal tolerance

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Abstract

Ailanthus altissima, a fast-growing and contamination-resistant species is investigated for its use in areas contaminated by heavy metals. A micropropagation protocol for *A. altissima* was developed, cultured shoots were tested for *in vitro* heavy metals tolerance. Proliferation rate and shoot length were affected by 6-benzylaminopurine (BAP) and Murashige and Skoog's (MS) salt concentrations, best results were obtained in full strength MS medium supplemented with 1.32 or 2.64 μM BAP. Rooting percentage was strongly influenced by indole-3-butyric acid. Cultures of *A. altissima* exposed to heavy metals demonstrated a tolerance comparable to species already utilized in phytoremediation.

Additional key words: shoot culture, copper, zinc, manganese, phytoremediation.

Ailanthus altissima Swingle is a world-wide spread tree with rapid growth rate and substantial reproductive ability that frequently out-competes native vegetation (Knapp and Canham 2000). The species is also described as a characteristic woody pioneer of disturbed sites, being able to grow in hard packed soils and polluted atmospheres (Pan and Bassuk 1986). The above observations suggest that *Ailanthus* could be employed in the reforestation of polluted areas where the introduction of other pioneer species has failed or in phytoremediation techniques.

Some species of the genus *Ailanthus* has already been used for tissue culture, *e.g. A. triphysa* (Natesha and Vijayakumar 2004), *A. malabarica* (D'Silva and D'Souza 1992) and for callus culture *A. altissima* (Zenkteler and Stefaniak 1991), but as yet no reports are available on the shoot culture of *A. altissima.*

Plant tissue culture techniques have previously been found useful in the selection of metal tolerant plants (Watmough and Dickinson 1995, Rout *et al.* 2005)

Explants obtained from shoots of a vigorous *Ailanthus altissima* Swingle tree, were washed for 10 min in water containing liquid detergent, surface sterilized by immersion (10 min) in a 50 % solution of commercial bleach, then rinsed 3 times in sterile distilled water. Explants were placed into test tubes containing Shenk and Hildebrandt (1972; SH) medium supplemented with 0.5 μM indole-3-butyric acid (IBA), 0.44 μM 6-benzyl-

aminopurine (BAP), 100 mg dm^{-3} myo-inositol, 0.4 mg dm^{-3} thiamine, 30 g dm^{-3} sucrose, 5.5 g dm^{-3} agar (*B&V*, Parma, Italy). Medium pH was adjusted to 5.7 with 0.1 M KOH and autoclaved for 20 min at 121°C and 138 kPa. Cultures were incubated in walk-in chambers at 23 ± 1 °C and 16-h photoperiod. Light was provided by cool white fluorescent lamps at photon fluence rate of 90 ± 10 μmol $m^{-2} s^{-1}$ at plant height.

After an initial growth, sterile shoots were transferred to *G7 Magenta* boxes (*Magenta Corporation*, Chicago, IL, USA.) containing 70 cm^3 of basal medium (BM) consisting of full strength Murashige and Skoog (1962; MS) salts supplemented with 0.5 μM IBA, 4.4 μM BAP, 100 mg dm^{-3} myo-inositol, 0.4 mg dm^{-3} thiamine, 30 g dm^{-3} sucrose, 5.5 g dm^{-3} agar. Subcultures were taken at monthly intervals.

One-node stem segments, 15 mm long, were placed horizontally in *G7 Magenta* boxes containing media derived from BM with different concentrations of MS salts (full, half and quarter strength), BAP (0, 1.32, 2.64 and 5.28 μM) and IBA (0 and 0.5 μM). After three weeks the proliferation rate and the length of new shoots were recorded.

To determine rooting ability, shoots were transferred to half strength MS media without cytokinins. Media were supplemented with 0, 1.5, 3, 6, 12 μM IBA. *Phytagel* (*Sigma*, St. Louis, USA) at a concentration of 2 g dm^{-3} was used as gelling agent. Microcuttings, 15 mm long, were placed vertically in test tubes containing 12 cm^3 of medium and left for three weeks under the same growth conditions used for shoot proliferation, the number and length of roots were then recorded.

In order to evaluate *in vitro* heavy metal tolerance of *A. altissima*, shoots 15 mm long, derived from subcultures, were placed vertically in *G7 Magenta* boxes containing the medium which showed the best performances in terms of proliferation rate (full strength MS salts; 1.32 μM BAP, 100 mg dm^{-3} myo-inositol, 0.4 mg dm^{-3} thiamine, 30 g dm^{-3} sucrose, 5.5 g dm^{-3} agar) supplemented with various concentrations of manganese ($MnSO_4 \cdot 1 H_2O$, 0.1 mM normal content in MS medium (control), 0.2, 0.4, 0.6, 0.8 and 1.6 mM) zinc ($ZnSO_4 \cdot 7 H_2O$, 0.03 mM normal content in MS medium (control), 0.06, 0.12, 0.24, and 0.48 mM) and copper ($CuSO_4 \cdot 5 H_2O$, 0.0001 mM normal copper content in MS medium (control), 0.02, 0.05, 0.1 and 0.2 mM).

Shoot proliferation and metal tolerance experiments were conducted using a completely randomized statistical design. Differences regarded as significant had a *P* value of less than 0.05 as determined by analysis of variance using the *Proc. GLM* procedure of *PC SAS* version *9.3.1* (SAS Institute, Cary, NC, USA).

Significant differences in terms of new shoot formation and shoot lengths were found among media with different MS salt concentrations: decreasing MS salts from full strength to half and quarter strengths caused a significant decline in the parameters observed (Table 1). Media with full strength MS salts turned out, therefore, to be the most appropriate for *in vitro* propagation of *A. altissima* confirming the results obtained for *A. triphysa* (Natesha and Vijayakumar 2004). Shoot development was efficient at 1.32 μM and 2.64 μM BAP (Table 1). In micropropagation of *A. tryphysa* a comparable mean number of shoots was achieved with 4.4 or 8.8 μM BAP (Natesha and Vijayakumar 2004). The addition of 0.5 μM IBA to the cultures caused significant decreases in proliferation and length of shoots cultured in media with full and half MS salt strength (Table 1). The addition of the indole-3-acetic acid (IAA) did not affect cultures of *A. triphysa* (Natesha and Vijayakumar 2004).

Rising concentrations of IBA increased the percentage of rooted shoots from 7.8 % (without IBA) to 71.7 % (with 12 μM IBA). IBA did not affect the mean number of primary roots while significant root elongation was recorded only in media with 12 μM of IBA (Table 2).

The growth of shoot cultured on $CuSO_4$ containing media was reduced at concentrations higher than 0.02 mM. However, cultures tolerate copper well at 0.05 mM (85 % of new shoots and 69 % of shoot length when compared to control) (Table 3). Zinc in culture did not affect shoot number up to a concentration of 0.12 mM (94 % of control), the same concentration had a significant effect on shoot length (59 % of control). Higher contents of zinc induced significant reduction of growth (38 % of new shoots and 42 % of shoot length if compared with control) (Table 3). Negative effects of zinc or copper at comparable concentrations in *in vitro* cultures were already observed in *Holarrena antidysenterica* (Agraval and Sharma 2006) and aspen (*Populus tremula* × *tremuloides*) (Kalisova-Spirochova *et al.* 2004)

Table 1. Effects of different combinations of MS salt strength, BAP and IBA on shoots of *A. altissima* after three weeks of culturing. Means ± SE, $n = 90$. Values followed by a different

Abbreviations: BAP - 6-benzylaminopurine; IBA - indole-3-butyric acid; IAA - 3-indoleacetic acid; NAA - naphthalene-1-acetic acid; MS - Murashige and Skoog's (1962) medium; SH - Shenk and Hildebrandt's (1972) medium.

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letter are significantly different.

MS salts strength	BAP [µM]	IBA [µM]	Number of shoots [explant^{-1}]	Shoot length [mm]
1	0	0	1.39 ± 0.06 defg	6.53 ± 0.41 e
1	0	0.5	1.31 ± 0.08 efgh	6.51 ± 0.48 ef
1	1.32	0	1.92 ± 0.08 a	17.00 ± 1.01 a
1	1.32	0.5	1.54 ± 0.10 cde	14.10 ± 0.86 b
1	2.64	0	1.87 ± 0.09 ab	16.30 ± 1.09 a
1	2.64	0.5	1.51 ± 0.11 cdef	16.71 ± 1.04 a
1	5.28	0	1.64 ± 0.10 bcd	13.03 ± 0.98 bc
1	5.28	0.5	1.18 ± 0.09 gh	10.90 ± 0.88 cd
0.5	0	0	1.28 ± 0.09 efgh	4.88 ± 0.38 fgh
0.5	0	0.5	1.34 ± 0.07 defgh	5.73 ± 0.39 efg
0.5	1.32	0	1.63 ± 0.09 bcd	15.11 ± 0.78 ab
0.5	1.32	0.5	1.62 ± 0.08 bcd	11.30 ± 0.63 cd
0.5	2.64	0	1.72 ± 0.08 abc	11.72 ± 0.89 cd
0.5	2.64	0.5	1.61 ± 0.10 bcd	10.81 ± 0.78 d
0.5	5.28	0	1.40 ± 0.09 defg	9.80 ± 0.58 d
0.5	5.28	0.5	1.51 ± 0.09 cdef	6.67 ± 0.53 ef
0.25	0	0	1.09 ± 0.08 hi	5.19 ± 0.44 efgh
0.25	0	0.5	1.30 ± 0.08 efgh	3.28 ± 0.25 h
0.25	1.32	0	1.23 ± 0.09 fgh	7.36 ± 0.68 e
0.25	1.32	0.5	1.57 ± 0.09 cde	6.45 ± 0.47 ef
0.25	2.64	0	0.90 ± 0.10 i	5.44 ± 0.69 efgh
0.25	2.64	0.5	1.31 ± 0.09 efgh	5.36 ± 0.49 efgh
0.25	5.28	0	0.63 ± 0.08 j	3.58 ± 0.35 gh
0.25	5.28	0.5	0.90 ± 0.09 i	3.89 ± 0.34 gh

Table 2. Rooting percentage, number of primary roots and primary root length after three weeks of culture on media supplemented with IBA. Means ± SE, n = 90. Values followed by a different letter are significantly different.

IBA [µM]	Rooted shoots[%]	Number of primary roots	Primary root length [mm]
0	7.8 ± 4.0 d	1.71 ± 0.58 a	65.43 ± 16.62 b
1.5	14.4 ± 4.8 cd	2.00 ± 0.52 a	43.98 ± 15.93 b
3.0	41.1 ± 15.7 bc	2.08 ± 0.21 a	47.62 ± 6.80 b
6.0	62.2 ± 2.2 ab	4.98 ± 0.63 a	61.84 ± 6.38 b
12.0	71.7 ± 5.0 a	5.73 ± 1.44 a	110.00 ± 10.61 a

Increasing concentrations of manganese in culture media caused a significant decline in morphogenesis, nevertheless shoots tolerate 0.4 mM of manganese in medium well (85 % of new shoots and 87 % of shoot length as compared to the control) (Table 3). Previous studies on manganese in culture media showed significant reduction of growth in callus lines of *Brassica campestris* and *B. juncea* (Rout *et al.* 2005).

The development of an efficient protocol of micropropagation and rooting for *A. altissima* was utilized for testing *in vitro* tolerance to heavy metals. *A. altissima* cultures show a tolerance comparable with that of cell lines obtained from *Acer pseudoplatanus* seedlings selected among trees surviving on metal contaminated soils (Watmough and Dickinson 1995). Zinc tolerance was similar to *in vitro* cultures of *Populus* (Kalisova-Spirochova *et al.* 2004), a genus already widely utilized in phytoremediation techniques (Rockwood *et. al* 2004). Soil pollution is often a result of the accumulation of several toxic metals as Cu, Zn, Pb, Cd, Hg, As, *etc*. (Alcantara *et al.* 2001), the evidence of the tolerance of *A. altissima* to multiple heavy metals as observed in this study suggests that this species can be tested for reforestation or phytoremediation of areas polluted with heavy metals.

Table 3. Number of new shoots and average shoot length in media supplemented with rising concentrations of heavy metals. Means ± SE, n = 90. Values followed by a different letter are significantly different.

Metals	Concentration [mM]	Number of shoots [explant^{-1}]	Number of shoots [%]	Shoot length [mm]	Shoot length [%]
Cu	0.0001 (cont.)	1.78 ± 0.09 a	100	12.44 ± 0.72 a	100
	0.02	1.97 ± 0.10 a	110	12.40 ± 0.74 a	99
	0.05	1.52 ± 0.10 b	85	8.57 ± 0.66 b	69
	0.10	0.80 ± 0.09 c	44	4.59 ± 0.49 c	37
	0.20	0.23 ± 0.05 d	12	2.29 ± 0.17 c	18
Zn	0.03 (cont.)	1.78 ± 0.09 a	100	12.44 ± 0.72 a	100
	0.06	1.88 ± 0.14 a	106	11.87 ± 0.81 a	95
	0.12	1.68 ± 0.08 a	94	7.36 ± 0.49 b	59
	0.24	1.27 ± 0.08 b	71	5.35 ± 0.36 c	43
	0.48	0.68 ± 0.08 c	38	5.24 ± 0.51 c	42
Mn	0.10 (cont.)	1.78 ± 0.09 ab	100	12.44 ± 0.72 a	100
	0.20	1.83 ± 0.10 a	103	11.90 ± 0.73 a	96

0.40	1.53 ± 0.10 b	85	10.80 ± 0.82 ab	87
0.80	1.18 ± 0.11 c	66	5.48 ± 0.48 b	44
1.60	0.48 ± 0.08 d	26	8.47 ± 1.62 c	68

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