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Full Length Research Paper

Influence of phytohormones and medium on the shoot regeneration from leaf of *Swertia chirata* Buch.-Ham. Ex wall. *in vitro*

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A procedure for *in vitro* shoot regeneration was formed from leaves derived from the field grown *Swertia chirata* and the effects of phytohormones and medium on the regeneration of shoot were tested by an orthogonal experiments. The best result was obtained in the 190-2 medium with 13.32 μM 6-benzylaminopurine and 0.54 μM α-naphthaleneacetic acid. The result of orthogonal analysis of variance showed that 6-benzylaminopurine and α-naphthaleneacetic acid significantly affected on the shoot regeneration. The propagation rate was 7.75 ± 3.46 when the shoots were cultured on the multiplication medium. The highest rate of shoots was 96.50% on the medium with 5.40 μM α-naphthaleneacetic acid. Histological studies showed shoot regeneration took place by somatic embryogenesis.

Key words: *Swertia chirata,* adventitious shoots, histology, an orthogonal test, leaves.

INTRODUCTION

*Swertia chirat*a, a gentian species, is an annual/biennial medical herb growing in the Himalaya from Kashmir to Bhuton and Khasi hills at altitude of 1000 - 3500 m. Traditionally, *S. chirata* has been used in Tibetan and Ayurveda medicines for febricity, stomachic and liver disorders. Extracts of *S. chirat*a emerged effective activities of antioxidative, antihepetotoxic and hypoglca-cemic active-ties and amarogentin rich in *S. chirat*a can reduce hyper-proliferation (Kar et al., 2003; Reen et al., 2001; Tripathi et al., 2005; Saha et al., 2006). The whole plants contain gentianine alkaloids and aerial part contains xanthones (Sharma, 1982). 63 compounds of oil were identified in the *S. chirata* by capillary GC-MS. Conventionally, *S. chirat*a is propagated through seeds. Low seed viability and germination percentage are the factor that discourage the larger scale commercial plantations of the herb. Over-harvesting from wild beyond the sustainable limit of *S. chirat*a and destruction of the plant's habits has resulted in the herb is endangering. *In vitro* plant regeneration is one of the most useful technologies to micropropagate herb. Micropropagation of *S. chirat*a *in vitro* has been reported by different labs. Wawrosch et al. (1999) reported that shoot regeneration was succeed from roots explants of *S. chirata in vitro*, limits of hyper-hydration and size of adventitious shoots exited in their experiments. Ahuja et al. (2003) patented shoot multiplication from nodal explants of *S. chirata*. Axillary multiplication shoot were induced from nodal explants derived from 4-week-old seeding and wild plants of *S. chirata* respectively and the genetic stability of regeneration plants were investigation using ISSR marker assay or RAPA-DNA fingerprinting pattern (Joshi et al., 2007; Chaudhuri et al., 2007; Joshi et al., 2007; Balaraju et al., 2009). Chaudhuri et al. (2008) have recently re-

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Abbreviations: ANOVA, Analysis of variance; **6-BA**, 6-benzyladenine; **NAA,** a-naphthaleneacetic acid; **MS,** Murashige and Skoog basal medium; **B5**, B5 basal medium; 190-2, 190-2 basal medium.

Table 1. Effects of different combinations of hormones and basal media on the adventitious shoots regeneration from leaves explants of *S.chirata*

6-BA(μM)	NAA(μM)	Basal media	%Explants forming shoots*	Number of shoots/ Regeneration explants*
4.44	0	MS	0.00 ± 0.00 [c]	0.00 ±0.00 [d]
4.44	0.54	B5	10.61 ± 1.32 [b]	2.20 ±0.37 [ab]
4.44	1.07	190-2	6.21 ± 4.08 [b]	1.45 ± 0.49 [c]
8.88	0	190-2	9.00 ± 3.60 [b]	1.95 ± 0.32 [ab]
8.88	0.54	MS	22.85 ± 5.12 [a]	2.65 ± 0.78 [b]
8.88	1.07	B5	9.48 ± 2.92 [b]	2.70 ± 0.57 [bc]
13.32	0	B5	7.18 ± 0.72 [b]	3.05 ± 0.21 [ab]
13.32	0.54	190-2	25.13 ± 2.54 [a]	4.30 ± 1.13 [a]
13.32	1.07	MS	21.53 ± 0.98 [a]	3.20 ± 0.85 [ab]

*Data represent mean ± SE. Means within a single column followed by the same letter were not significantly different according to ducan's multiplication range test at the 5% level.

ported shoot directly regenerated from leaves taken from *in vitro* shoot culture.

The present investigation described the adventitious shoots regeneration from leaves explants derived from the field grown plant. The effects of plant growth regulators and medium on shoots formation were investigation by an orthogonal experiment and examined the advanced stages of shoot formation by histological studies.

MATERIALS AND METHODS

Plant material

The leaves obtained from the shoots of *S. chirata* growing in field were surface sterilized in a solution of 0.1% $HgCl_2$ for 7 min and washed with sterile water 5 times. The sterilized leaves were excited into 1 cm length and planted on the dish with medium supplemented with different concentration and combination of NAA and 6-BA.

Medium and culture condition

An orthogonal experiment including 3 factors and 3 levels was projected to study the effects of phytohormone and basal media on the shoot induction of *S. chirata*. The basal medium included MS (Murashige and Skoog, 1962), B5 (Ganborg et al., 1968) and 190-2 (Zhuang et al., 1984). These medium were supplemented with different concentration of NAA (0, 0.54, 1.07 μM) combination with 6-BA (4.44, 8.88, 13.32 μM), respectively (Table 1). The pH of all medium was adjusted to 5.8 and solidified with 5 g l^{-1} agar and the media were autoclaved at 121℃ for 20 min. Explants were placed upside down on the 90 mm diameter Petri dish containing 25 ml inducing medium. Cultures were grown in the dark at 22 ± 2℃ for 4 weeks. Then the cultures were maintained under a photoperiod of 16/24 of light (116 μmol m^{-2} s^{-1}). All explants were subculture on the fresh medium every 4 weeks.

Multiplication and rooting of shoot

The adventitious shoots were transferred on the multiplication medium which was the MS basal media supplemented with 13.32 μM 6-BA. When cultured for 4 weeks the numbers of propagation shoots per shoot were investigated. A 2-step method was used in the roots induction of regeneration shoots. Firstly, elongated shoots were excised and incubated on the MS medium without any plant regulators. After 2 weeks, shoots were subculture on the medium supplemented with different concentration of NAA (0, 1.07, 2.70, 5.40 μM) alone or in combination with 6-BA (0.44 μM) to induce roots.

Histology

For histological examination, leaf explants at different culture stages were fixed in FAA (formalin:acetic acid:alcohol, 1:1:18), dehydrated through a graded ethanol series for 2 h in each step and embedded in paraplast. Serial sections were cut 10 um thick, stained in safranin-fast green, mounted in DPX. Samples were photographed using NIKON light microscope.

Statistical analysis

All experiments were carried out at least 20 explants in each treatment and repeat twice. The data of shoots regeneration were subjected to an orthogonal analysis of variance for the effects of medium and phytohormone in the shoot induction. The rates of rooting of shoots on the medium were analyzed by 1-way ANOVA. The significant differences between the means were assessed by Duncan's multiple range test at $P = 0.05$.

RESULTS AND DISCUSSION

Organogenesis from leaf explants

Embryogenic callus were observed on the edge or incision of explants in all treatments after 4 weeks (Figure 1A). The callus became green when the cultures were incubated in the light. After 6 weeks culture, somatic embryos and germination of somatic embryo occurred on the explants, root occurred simultaneously from same explants on several media (Figure 1B). With the further cul-

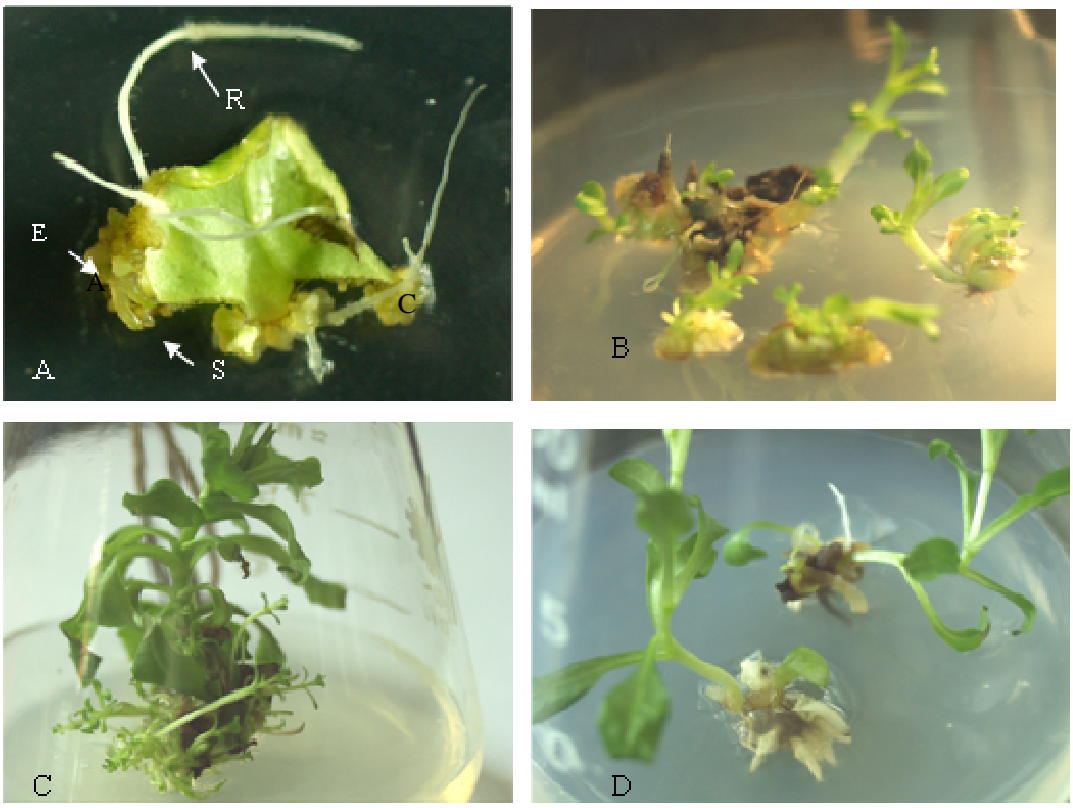

Figure 1. Adventitious shoots formation from leaves of *S. chirata*. (A) Somatic embryo (E) adventitious shoots (S) and roots (R) simultaneously formated from leaf explants. (B) Adventitious shoots regeneration from. (C) Adventitious shoots propagated in the multiplication medium. (D) Rooting of shoots on the rooting medium.

ture, adventitious shoots developed on different media (Figure 1C). The frequency of regeneration of shoots was significantly different on the 9 media (Table 1). Neither shoot nor root was induced on the MS basal medium with 6-BA 4.44 μM alone. The best result of shoot regeneration was achieved on 190-2 basal medium containing 13.32 μM 6-BA and 0.54 μM NAA. The next higher rate of shoot regeneration was 22.85 ± 5.12% in the MS media supplemented with 6-BA 8.88 μM and NAA 1.07 μM, and the followed was 19.23% which was obtained in the MS medium with 6-BA 8.88 μM and NAA 0.54 μM. At the same time, roots were induced but the frequency of root induction was low or zero in the media (data not shown).

Effects of plant regulators and medium on induction of shoots

The result of orthogonal analysis of variance showed the 6-BA and NAA significantly affected on the shoot regeneration ($F = 29.64$, $p < 0.05$; $F = 26.87$, $p < 0.05$ respectively). High concentrations of 6-BA was benefit to shoot induction. According to previous studies, 6-BA was a key factor to induce shoots regeneration and enhanced shoot formation *in vitro* (Chandra and pal, 1995; Ma, 1998). It was noted that the rates of shoots regeneration were low or zero on the media without anxin (NAA), while NAA (0.54 μM) was added in the medium could enhanced shoot induction. The result showed NAA has important effect for the shoot organogenesis of *S. chirata*. Studies had confirmed that auxin was a key factor for inducing embryogenic cell and can affect somatic embryo development and morphology (Ma and XU, 2002; Mandal and Gupta, 2003). Our result coincided basically with previous study on the shoot formation using leaves taken from *in vitro* shoot culture of *S. chirat*a (Chaudhuri et al., 2008). The frequency of shoots regeneration was affected by the concentration and combination of 6-BA and NAA and the number of shoots per explants was affected by 6-BA/NAA interaction in the study. This may suggest a synergestic effect of 6-BA and NAA increased shoot morphogenesis that has been shown in other plants (Amutha et al., 2003; Deepark et al., 2005). Studies have been reported that the medium composition affected embryo production and regeneration (Samson et al., 2006; Zhang et al., 2008; Jain et al., 2008). However, the basal medium did not significantly affected on the shoots formation ($F = 4.97$, $p > 0.05$) in our investigation

Table 2. The percentages of shoots rooting on medium with different hormones.

NAA (μM)	BA (μM)	Percent of rooting (%)*	Number of roots per shoot*
0	0	55.77 ± 8.16 [b]	3.65 ± 1.03
1.07	0	83.00 ± 9.90 [a]	4.56 ± 1.25
2.70	0	91.67 ± 3.93 [a]	5.47 ± 2.00
5.40	0	94.57 ± 2.80 [a]	4.81 ± 1.27
1.07	0.44	85.52 ± 4.77 [a]	5.80 ± 1.05
2.70	0.44	74.60 ± 4.49 [ab]	5.24 ± 1.22
5.40	0.44	83.15 ± 18.59 [a]	4.32 ± 1.08

*Data represent mean ± SE. Means within a single column followed by the same letter were not significantly different according to ducan's multiplication range test at the 5% level.

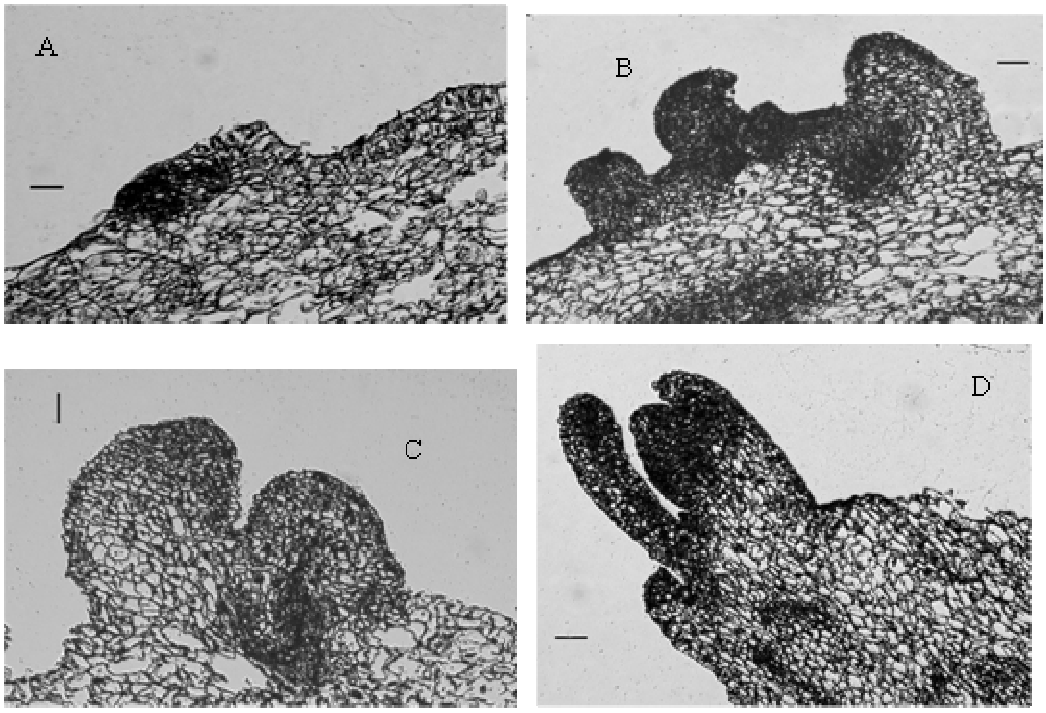

Figure 2. Histology of somatic embryogenesis and organogenesis from leaf explants of *Swertia chirata.* (A) The meristematic domes appeared on the suiface of the exolant. Bar = 92 μM. (B) Globular structure and globular embryo development. Bar = 92 μM. (C) cotyledonary embryo at 6 weeks of culture inition. Bar = 92 μM. (D) Adventitious shooots with differentiated primary leaves and apical meristems. Bar = 92 μM.

by the analysis of orthogonal analysis. In a word, the medium supplemented with 6-BA in combination with NAA (low concentration) would be benefit to shoot formation.

Multiplication and elongation of shoots

The induction shoots were transferred to the multiplication medium that grew fast and propagated on this medium (Figure 1D). The mean number of multiplication per shoot was 7.75 (SE ± 3.46) and no hyperhydration was observed. Hyperhydration of adventitious shoots generally occurred on all media in the report about shoots regeneration from root explants (Wawrosch et al., 1999). The hyperhydration of adventitious shoots might be due to the explants type.

Rooting of shoots

Adventitious shoots were separated single and transferred into the new bottles with MS medium without any hormones. 2 weeks later the shoots were subculture on

the MS medium supplemented with NAA (0, 1.07, 2.70 and 5.40 μM) alone or in combination with 6-BA (0.44 μM) for rooting. The greatest percentage of shoot rooting occurred on the medium containing 5.40 μM NAA (Table 2). 1-way analysis of variance showed the frequency of root was significantly different ($p < 0.05$), but there was not significantly different among the number of root per shoot on different medium.

Histology of shoot organogenesis from immature leaves

Histological studies were carried at different stages of shoot development. After 2 weeks of culture, cell division occurred in the cell of the adaxial epidemal and in the palisades. Subsequent division resulted in formation of new meristematic center along the epidermis (Figure 2A). At 4 weeks, the meristematic domes were formed on the surface of explants. With the prolongation of culture time on the induction medium, globular shaped structures and globular embryos were observed (Figure 2B). Their further development led to cotyledons embryo after 6 weeks (Figure 2C). The embryo differentiated to adventitious shoot and the shoot had differentiated primary leaves and apical meristem (Figure 2D).

Conclusions

In our experiments, shoots were induced from the leaves taken from fields. The best results were obtained on the media contained 6-BA 13.32 μM and NAA 0.54 μM. The effects of 6-BA, NAA and medium were studies in plantlet regeneration from leaves of *S. chirata*. The 6-BA and NAA significantly affected on the shoot regeneration and hyperhydration shoots was not occurred in this investigation. The percentages of shoot rooting ranged from 61.54 to 96.55% on different rooting media. The system of shoot regeneration from mature leaves of shoots growing on the field will be used to genetic transformation experiments of *S. chirata* in further works.

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