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# Assessment of Secondary Metabolites in *In vitro* Regenerated Plantlets of *Oroxylum indicum* (L.) Vent

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## Abstract

Flavonoids baicalein and baicalein-7-o-glucoside were estimated in *in vitro* raised (six months old) as well as nature grown (12 months old) plants of *Oroxylum indicum* (L.) Vent. Multiple shoot formation and elongation were obtained from axillary and apical bud explants of *O. indicum* on MS containing BAP (1 mg/l) and $AgNO_3$ (2 mg/l). Rooting of the regenerated shoots was achieved on medium (half strength) containing IBA (1 mg/l) and $AgNO_3$ (1 mg/l). A quantitative HPLC analysis of methanolic extract of leaf and root indicated the enhancement of baicalein-7-o glucoside in *in vitro* raised root and leaf of *Oroxylum indicum.*

## Introduction

Since time immemorial medicinal plants have been used virtually in all cultures as a source of medicine (Kumar 2004, Patwardhan et al. 2004). The production, consumption and international trade in medicinal plants and phytomedicine (herbal medicine), have grown and are expected to grow further in the future. To satisfy growing market demands, surveys are being conducted to unearth new plant sources of herbal remedies and medicines and at the same time develop new strategies for better yield and quality. This can be achieved through different methods including micropropagation (Dubey et al. 2004). It may help in conserving many valuable tree species in the process and may open new vistas in the forest biotechnology.

*Oroxylum indicum* (L.) Vent. (Bignoniaceae) commonly known as Shivnak, Shyonak, Sonpatha or midnight horror, is a small deciduous, soft wooded tree. It is distributed throughout the country up to an altitude of 1200 m and found mainly in ravine and moist places in the forests (Bennet et al. 1992).

Several parts of this tree contain alkaloids and flavonoids (Grampurohit et al. 1994, Chen et al. 2003) of medicinal value. The plant is used in many ayurvedic preparations widely used by people for health care. The important

medicinal principles obtained from it are *shyonaka patpak* and *Bruhat pancha mulayadi kwath* (Yasodha et al. 2004). *Dashmula* is one of the best known health care products of Ayurveda. The main ingredients of *Dashamula* are procured from the roots of five herbaceous and five tree species, *shivnak* being one of them. This species also constitutes one of the ingredients in *Chyawanprasha* (Ghate 1999, Parle and Bansal 2006). Dichloromethane extracts of the stem bark and root possess antimicrobial, antifungal, anti-inflammatory and anticancerous properties (Ali et al. 1998, Lambertini et al. 2004).

## Materials and Methods

Seeds of *Oroxylum indicum* were collected from forest areas in and around Jabalpur. Seeds were germinated on sterilized moist filter paper. *In vitro* raised seedlings were given a treatment of 1 - 2 min each of 70% ethyl alcohol and 0.1% mercuric chloride. The explants *viz.* apical buds (ApB) (0.5 - 1 cm) and axillary buds (A x B) (0.7 - 1 cm) were dissected from 15 - 20 days old seedlings (8 cm). Explants were inoculated under aseptic conditions on to the sterile culture medium in test tubes on MS containing 3% sucrose, and plant growth regulators (PGRs) particularly cytokinins *viz.* BAP (1 mg/l) with additive *viz.* $AgNO_3$ in different concentrations (0.1, 1.0, 2.0, 4.0 mg/l). The medium was solidified in 0.7% agar. The cultures were maintained in culture tubes and conical flasks and were kept in the culture room at a temperature of 25 ± 2ºC, relative humidity of 60 - 70% and a light intensity of approx. 1500 lux provided by cool, white, fluorescent tubes under a photoperiod of 16/8 h (light/dark).

The effect of continuous supplementation of PGRs on direct shoot regeneration was observed up to three subculture passages each of 20 - 22 days. All experiments were completely randomized and repeated at least twice. Each treatment consisted of 20 - 25 replicates.

*Secondary metabolites:* The tissue culture generated plants, were hardened (Gokhale and Bansal 2009). Hardened plants were grown in soil in natural conditions for six months. Nature grown (one-year-old) and *in vitro* regenerated hardened plants (six months) were used for this purpose.

*Extraction of plant sample:* In this study 1 g each of leaves, roots obtained from one-year-old nature grown plants, callus, *in vitro* raised root and leaf obtained from six months old plantlets were extracted in Soxhlet apparatus for 7 h daily for three days with 300 ml of 70% methanol. The extract was then filtered with Whatman's filter paper No. 1 and evaporated on hot plate at 30°C. The residue was redissolved in 50 ml water and extracted three times with 75 ml 2-butanol. The 2-butanol layer was evaporated at a temperature of 30°C on hot plate. The extract was obtained in form of a yellow colored complex, which was subjected to HPLC analysis.

*HPLC analysis:* HPLC analysis of flavonoids was determined by the method of (Chen et al. 2003). The fraction analysis was carried out in a Hewlett-Packard 1100 HPLC. A Hypersil $C_8$ RP column (150x 4.6 mm I.D.) was used at a temperature of 30ºC, a flow rate of 1.0 ml/min and with a detection wavelength of 275.5 nm. The solvent system was used with gradient elution: 0 - 15 min 0.2% formic acid from 80 to 35% and acetonitrile from 20 to 65%; 15 - 20 minute 0.2% formic acid from 35 to 10% and acetonitrile from 65 to 90%.

## Results and Discussion

Shoot regeneration from embryonic axis explant (both direct regeneration as well as through callus) has been studied previously in *O. indicum* (Dalal and Rai 2004, Gokhale and Bansal 2005, Gokhale and Bansal 2008).

From Table 1 it can be observed that supplementation of both additives with SM (selected media) (MS with BAP 1 mg/l) resulted in high efficiency of multiple shoot induction without interference of callus. From the two explants *viz.* apical bud (ApB) and axillary bud (A x B) the highest frequency of shoot

**Table 1. Effect of SM + $AgNO_3$ on shoot regeneration from different explants of *O. indicum.***

| $AgNO_3$ | FSI | | SN | | SL | |
|---|---|---|---|---|---|---|
| (mg/l) | ApB | AxB | ApB | AxB | ApB | AxB |
| 0 | 87.6 | 83.56 | 1 | 7 | 2 | 1.5 |
| 0.1 | 60.26 | 67.54 | 6.5 | 6.24 | 2 | 1.2 |
| 1 | 75.34 | 79.9 | 3.4 | 12.4 | 2.2 | 2 |
| 2 | 90.54 | 93.39 | 12.9 | 18.5 | 3.2 | 3 |
| 4 | 72.03 | 81.67 | 7.4 | 10.32 | 2.9 | 1.7 |

SM = Selected media, FSI = Frequency of shoot initiation, SN = Shoot number, SL= Shoot length, ApB= Apical bud, AxB = Axillary bud.

initiation recorded were 90.54 and 93.39, shoot number (SN) 12.25 and 18.5 (Figs.1-4, 5-8) and shoot length (SL) 2.2 and 2.5, respectively on MSM + BAP 1 mg/l + $AgNO_3$ 2 mg/l. Shoots were separated from multiple shoot buds in at least three successive cycles. Effect of this combination persisted up to three cycles (Table 2). This combination of additives and PGR proved best for shoot multiplication (Figs. 9-10). Drastic reduction in shoot regeneration were observed with increasing or decreasing concentrations of $AgNO_3$. Exogenous supply of $AgNO_3$ efficiently blocked the production of ethylene (Beyer 1976). Several reports clearly demonstrate that the addition of $AgNO_3$ in the culture medium significantly enhances organogenesis (Purunhauser et al. 1987, Kumar and Pratheesh 2004, Pati et al. 2004).


Figs. 1-4. Multiple shoot regeneration from AxB. 5-8. Multiple shoot regeneration from ApB. 9-10. Elongated multiple shoots in *in vitro*. 11-13. Rooting of *in vitro* regenerated shoots. 14. Acclimation and hardening of *in vitro* regenerated shoots in water. 15. *In vitro* regenerated plantlets.

Various auxins have been known to possess different potential for ethylene production as well as callus formation at the basal portion of shoots. In the present study addition of $AgNO_3$ (1.0 mg/l) with selected medium for rooting (IBA 1 mg/l with half strength of MS) produced efficient healthy root systems with proper shoot growth (Table 3, Figs. 11-13). There are some reports where $AgNO_3$ has been used to inhibit callus formation during rhizogenesis in *Garcinia mangostana* (Chongjin et al. 1997), *Albizia procera* (Kumar et al. 1998) and *Cassava* (Zhang et al. 2001)

**Table 2. Effect of SM+$AgNO_3$ on shoot number (SN) and shoot length (SL) in *O. indicum* ( up to three subculture passages).**

| | SN | | | | | |
|---|---|---|---|---|---|---|
| $AgNO_3$ | A × B passage | | | ApB passage | | |
| (mg/l) | I | II | III | I | II | III |
| 0 | 7.8 | 12.7 | 14.3 | 1 | 5.7 | 6.5 |
| 0.1 | 7.2 | 9.4 | 5.2 | 6.5 | 4.2 | 8.4 |
| 1 | 10.5 | 12.5 | 15.7 | 3.4 | 7.3 | 10.4 |
| 2 | 22 | 26.8 | 28.9 | 12.9 | 16.2 | 20.7 |
| 4 | 6.8 | 4.6 | 2.1 | 7.4 | 4.6 | 3.4 |
| | | | SL | | | |
| 0 | 1.5 | 1.5 | 1.5 | 2 | 2 | 3 |
| 0.1 | 1.2 | 1.5 | 1 | 2 | 2.5 | 2.5 |
| 1 | 2 | 2.5 | 3 | 2.2 | 2.6 | 2 |
| 2 | 3 | 3.5 | 4 | 3.2 | 3.5 | 4.5 |
| 4 | 1.7 | 2 | 2.2 | 2.9 | 2.8 | 3 |

SM = Selected media, SN = Shoot number, SL= Shoot length, ApB = Apical bud, AxB = Axillary bud.

**Table 3. Effect of SM+$AgNO_3$ on rooting from *in vitro* regenerated shoots of *O. indicum*.**

| $AgNO_3$ (mg/l) | FR | | | NR | | | RL | | |
|---|---|---|---|---|---|---|---|---|---|
| | Media strength | | | Media strength | | | Media strength | | |
| | F | H | Q | F | H | Q | F | H | Q |
| 1 | 91.73 | 73.24 | 81.57 | 17.41 | 19.52 | 15.74 | 5.2 | 6.2 | 5 |
| 2 | 73.62 | 68.47 | 60.38 | 21.36 | 18.24 | 9.32 | 5.5 | 5 | 3.2 |

SM = Selected media, FR = Frequency of rooting, NR = No. of roots, RL = Root length, F = Full, H = Half, Q = Quarter.

The shoots could be readily rooted with a high frequency on half MS as reported earlier (Saha et al. 2003, Rajore and Batra 2005). MS (half strength) has been found resulting in healthy, strong and efficient root systems in the present work too.

In order to acclimatize, the plants were initially kept in distilled water in conical flasks (Eight days, Fig. 14) and were then transferred to soil : sand (1 : 1)

in polybags covered with polythene to maintain high humidity (Parveen et al. 2006). Such plants were transferred to earthen pots (Fig. 15) after a period of eight - ten days, irrigated regularly and then planted in the field.

The HPLC analysis of the extract of regenerated plant was carried out to check the percentage Baicalein quantitatively. A sharp peak for standard Baicalein was obtained at Rt 4.78 peak for another compound Baicalein-7-o-glucoside was obtained at Rt 6.11 (Chen et al. 2003). A comparative analysis of the results is given in Table 4.

**Table 4. Flavonoid contents of different plant parts and callus.**

| No. | Plant parts | Baicalein (%) | Baicalein–7-o-glucoside (%) |
|---|---|---|---|
| 1 | *In vitro* leaf | 0.93 | 50.29 |
| 2 | *In vitro* root | 4.25 | 21.33 |
| 3 | Nature grown leaf | - | 17.49 |
| 4 | Nature grown root | 6.19 | - |

Flavonoid baicalein (4.25%) was observed in the roots of *in vitro* raised plantlets (Chromatogram-3). Whereas, the highest percentage of baicalein-7-o-glucoside (50.29%) was obtained from the leaves of *in vitro* raised plantlets (Chromatogram-2). Analysis of nature grown plant's leaves and root sample showed 17.49% of baicalein-7-o-glucoside and 6.19% baicalein, respectively (Table 4).

*In vitro* regenerated plantlets have been reported to produce higher yields of active compounds (Fowke et al. 1994, Han et al. 1999, Mahagamasekera and Doran 1998). The production of high yield of secondary compounds has also been reported from callus culture, from suspension culture or by using precursor (Cusido et al. 1999, Croteau et al. 2000, Jordan et al. 2006).

The data generated in the present study are expected to support micropropagation of *O. indicum*. However, there is a need to carry out more advanced phytochemical studies by using different protocols of micropropagation.

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