Optimization of in vitro and ex vitro regeneration and micromorphological studies in *Basella alba* L.

Mahipal S. Shekhawat¹ · M. Manokari¹

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Abstract The optimum concentrations of the plant hormones for in vitro regeneration and subsequent effect of auxins on rooting (in vitro and ex vitro) of shoots of *Basella alba* L. have been investigated in present study. Nodal shoot segments were used as explants to initiate the cultures. The bud breaking from explants was observed within 1 week of incubation on agar gelled Murashige and Skoog's (MS) medium. Multiple axillary shoots (7.30 ± 0.56 shoots per explant) were induced on MS medium supplemented with 2.0 mg/L 6-benzylaminopurine (BAP). The shoots were multiplied (maximum 17.10 ± 0.44 shoots per explant) on the same medium fortified with 0.5 mg/L each of BAP and Kin (Kinetin) +0.1 mg/L IAA. These shoots were excised and rooted in vitro (10.73 ± 0.92 roots per shoot) on half-strength MS medium augmented with 2.0 mg/L indole-3 butyric acid (IBA). Hundred percentage success rates have been achieved by ex vitro rooting of the in vitro regenerated shoots with IBA at 300 mg/L. The in vitro and ex vitro rooted shoots were acclimatized in greenhouse and subsequently transferred to the natural field conditions where 100 % survival rate was reported. The ex vitro rooting method was found more advantageous than in vitro rooting in terms of time, energy and survival percentage of *B. alba*. A comparative foliar micromorphological study of *B. alba* was conducted to understand the micromorphological changes in plants while shifting from in vitro to the in vivo conditions in terms of variations in stomatal index, venation pattern and vein density, and the arrangement of crystals.

The study could help in understanding the response of in vitro raised plants towards in vivo environment.

Keywords *Basella alba* · Regeneration · Ex vitro rooting · Hardening · Micromorphology

Abbreviations

MS	Murashige and Skoog's medium
BAP	6-benzylaminopurine
Kin	Kinetin
IAA	Indole-3 acetic acid
IBA	Indole-3 butyric acid
NAA	α -Naphthalene acetic acid
PFD	Photon flux density

Introduction

Basella alba L. (family *Basellaceae*) commonly known as Indian Spinach, Ceylone Spinach or Malabar nightshade is mostly cultivated as leafy vegetable. It is extremely heat tolerant plant, native of tropical Asia and originated from India or Indonesia (Bamidele et al. 2010). It is a succulent-branched climber and the tender shoots are used as a cool season vegetable. The fruits are the potential source of natural colorant. Anthocyanins are found responsible for different colors present in the body parts of *B. alba* (Maiti and Ritthiruangdej 2008). The natural maroon color obtained from the ripened fruit's saps of *B. alba* used in dyeing industry as natural dye in India (Ozela et al. 2007). The dye isolated from the fruit pulp of this plant is used as an alternative to crystal violet or safranin for the staining of microbes (Mundo et al. 1995). The purple colored extract from the fruit of *Basella* is also used in coloring of food items.

✉ Mahipal S. Shekhawat
smahipal3@gmail.com

¹ Biotechnology Laboratory, Department of Plant Science,
M.G.G.A.C., Mahe, Pondicherry 673311, India

Basella contains some unique compounds like, basel-saponins, flavonoid kaempferol, β -carotene, Betacyanin betalain etc. (Yang et al. 2008). The leaf extract is reported to possess vital vitamins A, C, E, K, folic acid, riboflavin, niacin, thiamine and minerals like calcium, magnesium and iron. The mucilage from *B. alba* has strong suspending ability along with high viscosity, and used as a good thickening agent in pharmaceutical and cosmetic industries. The gel obtained from the mucilage used as medicines for skin problems due to its antioxidant activity (Toshiyuki et al. 2001).

Basella alba is reported for its anti-fungal, anticonvulsant, antimicrobial, antipruritis, analgesic, anti-inflammatory, antipyretic, anti-ulcer and androgenic properties, and used in the treatment of hypertension, anemia, hemorrhages, skin diseases, sexual weakness, dysentery etc. (Haskell et al. 2004). The leaves and stem have blood building properties. In India the stem and leaves of *B. alba* is used in the traditional systems of medicines for the treatment of melanoma, leukemia and oral cancer (Premalatha and Rajgopal 2005). The cooked roots are used to treat diarrhea and dysentery. The mucilage obtained from the leaves is a traditional Thai medicine in the treatment of bruise, ringworm, laboring and skin irritation, and also used to treat freckle and acne in Bangladesh (Akhter et al. 2008). In Nepal the juice is used in the treatment of catarrh, dysentery, anemia, cold and cough, orchitis, epididymitis, spurious labor, urticaria, constipation and gonorrhea (Foch et al. 2009).

Basella alba is now cultivated for its industrial purposes and naturally propagated by the stem cuttings (Cyunel 1989), this method causes the carry-over of disease-causing pathogens from one generation to the next (Sankar et al. 2011). The propagation through seeds has some limitations due some special requirements in germination of seeds and proper flowering in this plant (Larkcom 1991). Plant tissue culture methods can be used for rapid multiplication of improved varieties and to produce disease free and quality planting materials (Kartha and Gamborg 1975). Some reports are available on the tissue culture and anthocyanin production using *B. alba* cells (Cyunel 1989; Guo and Bin 2001). However, efficient in vitro propagation protocol for this plant species is largely deficit in the literature. The plant growth regulators were tested and conditions were optimized for efficient shoots and roots development in *B. alba* during present investigation. In this study, we report an excellent root regeneration protocol using ex vitro rooting methods which could reduce the time and energy in production of *B. alba*.

Since, photosynthesis being the essential part of leaf metabolism, the study of foliar anatomy from in vitro to field environment could help to improve the establishment of plants in the soil (Yokota et al. 2007; Chirinea et al.

2012). The present study also aims to evaluate micromorphological developmental changes in the leaf from in vitro to in vivo environment.

Materials and methods

Collection of plant material and preparation of explants

Basella alba plants were collected from the Villianur area of Pondicherry, South India during the months of January to June, 2013. Fresh shoot sprouts were used as explants. The stems were cut into 3–4 cm long segments with 1–2 nodes. The explants were first treated with 0.1 % (w/v) Bavistin (a systemic fungicide) for 6–7 min, and surface sterilized with 0.1 % HgCl_2 (w/v) for 5–6 min with alternate shaking followed by washing with autoclaved distilled water for 5–6 times under aseptic conditions in a laminar air flow cabinet (Technico Ltd. Chennai, India).

Establishment of culture

The sterilized nodal segment were inoculated on agar gelled (0.8 % agar) MS medium (Murashige and Skoog 1962) supplemented with 6-benzylaminopurine (BAP) and/or kinetin (Kin) (ranging from 0.5 to 5.0 mg/L) for shoot bud induction. Explants with single and double nodes were inoculated vertically in each culture tubes for bud breaking from the nodal meristems. Cultures were incubated at $30\text{--}35\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$ PFD (photon flux density) light for shoot induction with 12:12 h light:dark photo period at $25 \pm 2\text{ }^\circ\text{C}$.

Multiplication of shoots in cultures

The shoots were multiplied by two methods (1) the original explants were repetitively transferred to fresh medium for 2–3 passages after harvesting in vitro raised shoots, and (2) the in vitro produced shoots were cut into 2–4 cm long segments (each with at least 1–2 nodes) and subcultured on fresh MS medium. The culture medium used for shoot multiplication was incorporated with various concentrations and combinations of cytokinins (BAP and Kin 0.1–1.0 mg/L) and indole-3 acetic acid (IAA) (0.1 mg/L). The multiple shoots were regularly subcultured on fresh medium after an interval of 4–5 weeks. Experiments were conducted to study the effect of liquid medium (MS medium without agar) on the rate of shoot multiplication. The cultures were kept in shaker incubator (Technico Ltd. Chennai, India) at 90 rpm without filter paper or any type of support.

Effect of auxins on rooting of shoots (in vitro)

Long (3–4 cm) and sturdy shoots were harvested individually from shoot clumps and transferred to half and full strength MS medium containing different concentrations of auxins (indole-3 butyric acid, IBA; IAA or α -Naphthalene acetic acid, NAA; 0.1–3.0 mg/L) with activated charcoal (100 mg/L). The cultures were incubated (1–2 days) under diffused light conditions ($20\text{--}25\ \mu\text{mol m}^{-2}\text{ s}^{-1}$ PFD) and after that under the normal conditions (as in establishment of the cultures).

Ex vitro root induction

To save time and energy in plantlet production, ex vitro root induction experiments were tried. Healthy and tall shoots (4–5 cm) were selected from in vitro cultures and harvested individually from shoot clumps. The cut ends of the shoots were dipped in auxins solutions (IAA, IBA or NAA) for 5 min. Different concentrations of auxins (100–500 mg/L) were prepared with help of distilled water. Finally, the pulse treated shoots were directly transferred to the bottles containing soilrite® (KelPerlite, Bangalore, India), moistened regularly with one-fourth MS salts and maintained in greenhouse for rooting and hardening together.

Hardening of regenerated plantlets

The rooted (in vitro) plantlets were rinsed with water, kept on a moist filter paper and transplanted immediately to the bottles containing autoclaved soilrite®. These plantlets were moistened with solution of one-fourth MS basal salts and shifted to greenhouse for the hardening of plantlets. After 30–35 days, the plantlets were transferred to nursery polybags and pots having perforation at in the bottom, containing potting mixture of sand, soilrite®, garden soil and organic manure (1:1:1:1).

Comparative foliar micromorphological studies of *B. alba*

Experiments were conducted to study the foliar micromorphological development of veins (vein density and venation pattern), and stomatal index (SI) of leaves under in vitro condition after 4th subculture of multiplication stage and after 6th week of field transferred plants. Plants were randomly selected between 3rd and 7th leaves for micromorphological experiments from both environments. The epidermal peelings were separated to study the changes in the structure and function of the stomata (Johansen 1940) and preserved in 50 % ethanol, and stained with 1 %

safranin (Loba chemie, India) aqueous solution. The stomatal index was calculated as per standard methods (Wallis 1985). The leaves were primarily fixed in formalin, acetic acid, ethyl alcohol in the ratio of 1:1:3 and cleared in 70 % ethanol (v/v) until the chlorophyll was totally removed, bleached in 5 % NaOH for 24–48 h, and rinsed in 4–5 changes of distilled water to remove bleaching agent to study the venation. The cleared leaves were then stained with safranin for 4–8 min and rinsed carefully in water to remove excess stain. Photomicrographs were taken with the digital camera mounted on the microscope (Labomed iVu 3100, USA).

Statistical analysis of the data

The experiments conducted with 20 explants for each treatment and repeated thrice. The results were subjected to analysis of variance and the significance of differences among mean values was carried out using Duncan's Multiple Range Test (DMRT) at $P < 0.05$ using SPSS software, version 16.0 (SPSS Inc., Chicago, USA).

Results and discussion

Selection of explants and establishment of cultures

Juvenile and light-purple-green colored explants were reported better in terms of percentage response with time (within 6–7 days) and used for culture initiation. The mature and dark purple colored explants were responded after 2–3 weeks of inoculation. It was observed that the juvenile explants were easy to sterilize (less contamination) with help of HgCl_2 as compared to mature explants. MS medium augmented with BAP (2.0 mg/L) was observed suitable for bud break from axillary meristems and produced maximum (7.30 ± 0.56) shoots (Fig. 1a, b). Kin was less responsive as compared to BAP for shoot induction and only 4.90 ± 0.67 shoots were regenerated on 2.0 mg/L Kin from each explant. Guo and Bin (2001) used cotyledon and hypocotyls as explants of *B. alba* and cultured these on MS medium. They observed callus from the explant surface, when MS medium was fortified with 6-BA alone or with NAA or 2,4-D. No callus was reported with the cytokinins as well as auxins during this study at any stage of the cultures. Cyunel (1989) established cultures for the production of pigments using *B. alba* cells. Effectiveness of BAP over Kin for shoot initiation from the buds has been reported in number of plant species like *Ceropegia bulbosa* (Phulwaria et al. 2013), *Leptadenia reticulata* (Rathore et al. 2013), *Turnera ulmifolia* and *Morinda coreia* (Shekhawat et al. 2014, 2015a).

Fig. 1 **a:** Bud breaking from the nodal region of explants. **b:** Multiple shoot induction. **c:** Multiplication of shoots after 2 week. **d:** Shoots multiplication after 4 weeks on agar gelled MS medium. **e** and **f:** Multiplication of shoots in liquid MS medium

Multiplication of shoots and maintenance of cultures

The shoots were multiplied by repetitive transfer of the original explants on the fresh medium after harvesting freshly induced buds. Maximum 17.10 ± 0.44 shoots were regenerated from each explant, when mother explants were repeatedly shifted to the fresh MS medium supplemented with 0.5 mg/L each of BAP and Kin + IAA at 0.1 mg/L (Fig. 1c, d). The shoot numbers and length were increased up to 3–4 passages with mother explants after that these were stabilized. The number and length of shoots were decreased (14.23 ± 0.26 shoots with 4.70 ± 0.23 cm length), when the concentrations of BAP and Kin increased from the optimal level. The inhibition of apical dominance in repetitive subculturing of the shoots could stimulate the basal dormant meristematic cells this lead to enhance the number of shoots regeneration in this stage (Phulwaria et al. 2013). The method of multiplication of shoots by repetitive transfer of mother explants has been reported in many plants (Sanchez et al. 1997; Panwar et al. 2012). The number of shoots produced in this study was significantly greater than those achieved in the earlier reports in *B. alba* (Cyunel 1989; Guo and Bin 2001) and *B. rubra* (Pumchaosuan and Wongroun 2009). Guo and Bin (2001) were able to regenerate maximum four shoots/explant from the

callus induced from hypocotyl explants on MS medium containing BA (2.0 mg/L) + NAA (0.3 mg/L). The synergistic effect of cytokinins and auxin in the medium could enhance the rate of shoots multiplication, and it has been reported by several researchers (Rathore et al. 2013; Patel et al. 2014a; Shekhawat et al. 2015b).

In vitro raised shoots were excised and subcultured in liquid MS medium for large scale multiplication of shoots. For this, the shoot clumps (made up of 3–4 shoots) were subcultured in liquid MS medium (without agar) augmented with 0.5 mg/L each of BAP, Kin and 0.1 mg/L IAA. The highest numbers of shoots (7–8 shoots per culture vessel) were regenerated on this medium composition (Fig. 1e, f). The present results are totally different from our earlier work on *Turnera ulmifolia* and *Morinda coreia* (Shekhawat et al. 2014, 2015a) where liquid medium was found excellent in multiplication of the shoots in cultures.

Auxins and in vitro root induction

The shoots were harvested from both agar gelled and liquid medium grown cultures for rooting experiments. The half strength salts MS medium containing 2.0 mg/L IBA with 100 mg/L activated charcoal was found optimum for in vitro rooting from the cut ends of the shoots. On this

medium, maximum number (10.73 ± 0.92) of roots with average length (8.79 ± 0.81 cm) was reported (Fig. 2a). Guo and Bin (2001) supports our finding in terms of strength of MS medium used for in vitro root induction. Rooting of regenerated shoots was reported in combination of half strength MS medium with 0.3 mg/L NAA by Guo and Bin (2001). Our result signifies that half strength of MS salts in medium is appropriate for in vitro rooting and supported by many authors (Rai et al. 2010; Patel et al. 2014b; Lodha et al. 2014). The activated charcoal in the rooting medium induces roots in vitro from the shoots because it helps in balancing the pH level and improves the absorption of nitrogen (Thomas 2008). Less percentage response (83 and 71 %) with lower root number (6.58 and 4.87) and root length (5.87 and 4.85 cm) were observed on medium supplemented with IAA and NAA respectively. In vitro rooted plants were acclimatized successfully under the greenhouse conditions (Fig. 2b–d). The efficacy of IBA over other auxins for root induction has been acknowledged in many plant species (Rathore et al. 2013; Patel et al. 2014a, b).

Ex vitro root induction and hardening of plantlets

Experiments were conducted to achieve rooting and hardening simultaneously using ex vitro method. Excellent results were obtained when the excised shoots were pulse treated with IBA solutions for ex vitro root induction. This is first report on the ex vitro rooting of the shoots of *B. alba*. Maximum response (100 %), number (8.77 roots per shoot) and length (4.63 cm) of roots were observed with IBA at 300 mg/L concentration. The lowest rooting response with 3.81 roots per shoot was recorded with IAA and the moderate results (4.77 roots per shoot) observed with NAA (Fig. 3a). Ex vitro root induction was successfully achieved in *Ceropegia bulbosa* (Phulwaria et al. 2013), *Leptadenia reticulata* (Rathore et al. 2013) and *Caralluma edulis* (Patel et al. 2014a), etc. This technique could save time, energy and labor which lead to the development of an effective in vitro plant propagation protocol. The plantlets raised using ex vitro rooting methods are more suitable to tolerate the in vivo stresses (Ranaweera et al. 2013).

Fig. 2 a: Induction of in vitro roots from the cut ends of the shoots. b–d: Different stages in hardening of in vitro rooted plantlets. e: Acclimatized plant in the field conditions

Fig. 3 **a:** Ex vitro rooting of the in vitro regenerated shoot. **b–e:** Different stages in the hardening of ex vitro rooted plantlets

The in vitro or ex vitro rooted plantlets were hardened in the greenhouse. Five mL of tap water was poured on alternate days when the plantlets were in bottles containing soilrite® in the greenhouse and then transferred to manure and soil containing polybags after 4–5 weeks (Fig. 3b–e). Water spray on daily basis was required for better survival rate during hardening of the plantlets in greenhouse. The rate of survival at the time of hardening was achieved maximum (100 %) during present study. Finally the plantlets were transferred to the natural conditions after another 2–3 weeks (Fig. 2e). This is the first report of ex vitro rooting in this plant species which favored the maximum survival rate (100 %) of *B. alba* under the natural environments.

Foliar micromorphological studies

The external morphology of the leaves from both the environments was similar. The epidermal cells were irregular, polygonal and slightly undulated. The density of epidermal cells was more under in vitro environment compared to the field transferred plants. Plants under in vitro environment possessed epidermal density of 132.7, which was reduced in the field environment (115.6) after transplantation. The paracytic (cruciferous) stomata were predominant but anisocytic stomata were rare. Stomatal abnormalities such as unopened stomata, stomata with one guard cell and stomata without guard cells were reported (Fig. 4a, b). Presence of paracytic and anisocytic stomata has been reported in *B. alba* by Adenegan and Akinnubi (2015). The in vitro plants presented 25.8 stomatal

apparatus, but 19.4 stomatal apparatus only observed in field transferred plants after 6 weeks. Stomatal index was decreased from in vitro (16.2) to field transferred plants (14.3), this may be due to the water stress after transplanting into the soil.

Venation was Brochidodromous type, veins and vein-islets were much developed, veinlets endings (veinlet terminations) were totally absent, aereoles (vein-islets) were polygonal and closed in field transferred plants; but very few in the in vitro leaves (Fig. 4c, d). Crystals were arranged along with the veins all over the lamina and frequent in field transferred plants, but less in the in vitro leaves (Fig. 4e, f). Ayomipo and Johnson (2015) also reported that the crystal druses are abundant and housed in the parenchyma cells. In the present study it was observed that ex vitro rooted plantlets showed little differentiation in leaf tissues when these were transferred to the field. Therefore, ex vitro rooting in the greenhouse enhanced the survival rate during hardening and field transfer of the plantlets.

Conclusion

An efficient in vitro propagation protocol has been developed for *B. alba*. Plant growth regulators, in vitro and ex vitro conditions were optimized for shoots and roots regeneration. All the shoots were rooted ex vitro in this study could save time, energy and labor in production of in vitro plantlets of this important medicinal plant. The foliar micromorphological studies of in vitro propagated

Fig. 4 Foliar micromorphological studies of *B. alba*. **a** and **b**. Stomatal pattern of in vitro raised leaves and field transferred plants. **c** and **d**. Venation pattern of the leaves of in vitro and in vivo plants. **e** and **f**. Arrangement of crystals in the epidermal cells of in vitro and field transferred plants

plantlets showed abnormal anatomical and physiological features were improved in the field transferred plantlets with decreased stomatal index and increased vein density.

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Compliance with ethical standard

Conflict of interest The authors declare no conflict of interest.

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