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Histochemical and Shoot Induction Studies of *Basella rubra L.* (Basellaceae)

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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 rubra L.* have been investigated in present study. Nodal shoot segments were used as explants to initiate the cultures. Histochemical studies of *in vitro* and *in vivo* plants revealed alkaloids, polyphenols and terpenoids concentrations were higher in *in vivo* plants. Ascorbic acid and tannin concentration have shown no difference in the content in *in vitro* and *in vivo* plants. MS medium supplemented with IBA 2.0 mg/l + NAA 0.5 mg/l + GA3 0.5 mg/l induced shoot regeneration MS+IBA 3.0 mg/l induced roots and the plant regeneration was achieved in MS + IBA 0.5 mg/l + GA3 1.5 mg/l + IAA 1.0 mg/l. SEM – EDX elemental analysis of *in vitro* plants have shown absorption of sodium, magnesium, silica, phosphorus, sulphur, chlorine and potassium from the medium. The *in vivo* plants have shown absorption of magnesium, silica, chlorine, potassium, calcium and iron.

Keywords: *Basella rubra*, SEM-EDX, Shoot induction.

Basella rubra L. (family *Basellaceae*) commonly known as Indian Spinach, is mostly cultivated as leafy vegetable. It is extremely heat tolerant plant, native of tropical Asia and originated from India (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 colourant. Anthocyanins are found responsible for different colours present in the body parts of *B.rubra* (Maisuthisakul and Ritthiruangdej 2008). The natural maroon colour obtained from the ripened fruit's saps of *B. rubra* 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.

Basella contains some unique compounds like, basella saponins, flavonoid kaempherol, ß-carotene and betacyanin betalain. (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.rubra* 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). Histochemical staining method was performed

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to locate phytochemicals such as alkaloid, ascorbic acid, polyphenols, tannins and terpenoids synthesized by *B.rubra* plant.

Basella rubra is now cultivated for its industrial purposes and naturally propagated by the stem cuttings (Cyunel 1989), this method causes the carryover 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.rubra* cells (Cyunel 1989; Guo and Bin 2001). However, efficient *in vitro* propagation protocol for this plant species is largely deficit in the literature. The histochemical and shoot induction studies by using plant growth regulators were tested and conditions were optimized for efficient shoots, roots and plant regeneration in *B.rubra* during present investigation of *in vitro* and *in vivo* plants and SEM-EDX elemental analysis of roots of *in vitro* and *in vivo* plants.

MATERIALS AND METHODS

Collection of plant material and preparation of explants

Basella rubra plants were collected from the green house, National college, Tiruchirapalli, Tamilnadu, India. 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 5 min, and surface sterilized with 0.1 % $HgCl_2$ (w/v) for 3 min with alternate shaking followed by washing with autoclaved distilled water for 3 times under aseptic conditions in a laminar air flow cabinet.

Establishment of culture

The sterilized nodal segment were inoculated on agar gelled (0.8 % agar) MS medium (Murashige and Skoog 1962) supplemented with IAA (Indole 3 acetic acid) ranging from 0.5 to 3.0 mg/l, NAA (á-Naphthalene acetic acid) ranging from 0.5 to 2.0 mg/l and GA3 (Gibberellic acid) ranging from 0.5 to 2.0 mg/l for shoot regeneration. MS + IBA (Indole -3- butyric acid) ranging from 0.5 to 3.0 mg/l for root induction, MS + IBA ranging from 0.5 to 3.0 mg/l and GA3 ranging from 0.5 to 3.0 + IAA 0.5 to 3.0 mg/l for plant regeneration. Explants with single node were inoculated vertically in each culture tubes for bud breaking from the nodal meristem. Cultures were incubated at 1500 lux light with 12:12 hr light: dark photo period at 25 ± 2 ^{0}C.

Multiplication of shoots in cultures

The shoots were multiplied by two methods (i) the original explants were repetitively transferred to fresh medium for 2–3 passages after harvesting *in vitro* raised shoots, and (ii) the *in vitro* produced shoots were cut into 2cm long segments (each with one node) and subcultured on fresh MS medium. The culture medium used for shoot multiplication was incorporated with various concentrations and combinations of IAA + IBA + NAA + GA3. The multiple shoots were regularly subcultured on fresh medium after an interval of 4–5 weeks.

Effect of auxins on rooting of shoots (*in vitro*)

2cm shoots were harvested individually and transferred to MS medium containing different concentrations of IBA + BAP. The cultures were incubated (1–2 days) under diffused light (800 lux) conditions and after that under the normal conditions (at 1500 lux).

Plantlet regeneration (*in vitro*)

The rooted shoots were transferred to MS + IBA + GA3 + IAA at various concentrations, incubated at 1500 lux light and plantlets were established in *in vitro* and were selected for hardening process after 45 days of culture.

Hardening of regenerated plantlets

The regenerated (*in vitro*) plantlets were rinsed with water, kept on a moist filter paper and transplanted immediately to the containers containing autoclaved soilrite. These plantlets were moistened with solution of one-fourth MS basal salt 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 Histochemical studies of stem anatomy

Histochemical studies were conducted to

compare the *in vivo* and *in vitro* plant stem anatomy for the phytochemicals like alkaloids, ascorbic acid, phenolic compounds, tannin and terpenoids. The thin hand sections were stained in different staining agents as per the procedure (Shanmugam *et.al* 2010). Photomicrographs were taken in the digital camera mounted on the Microscope Olympus, Magcam DC 10 with magnification 40X.

RESULTS AND DISCUSSION

The Table-1 reveals the comparative

Table 1. Phytochemical analysis of *in vivo* and *in vitro B.rubra.L* plant stem (anatomy) by histochemical studies

Histochemical parameters	*In vivo* analysis	*In vitro* analysis
TBO (control)		
Epidermis	Light blue	Light blue
Hypodermis	Greenish purple	Greenish purple
External cortex	Blue	Blue
Vascular region	Purplish blue	Purplish blue
Pith	Light blue	Light blue
Alkaloid		
Epidermis	Orange	Orange
Hypodermis	Brown	Red
External cortex	Orange	Red
Vascular region	Grey – Brown	Brown
Pith	Black	Yellow
Result	Golden yellow /Brown	Golden yellow /Brown
Ascorbic acid		
Epidermis	Light blue	Black
Hypodermis	Brown	Pinkish red
External cortex	Blue	Golden yellow
Vascular region	Dark blue	Golden yellow
Pith	Grey– Brown	Grey / Violet
Result	Black silver / Red brown	Black silver / Red brown
Polyphenols		
Epidermis	Grey	Yellow
Hypodermis	Green	Green
External cortex	Reddish brown	Green
Vascular region	Grey brown	Green
Pith	Whitish grey	Grey
Result	Cherry red	Cherry red
Tannin		
Epidermis	Pink	Pink
Hypodermis	Green	Green
External cortex	Grey	Grey
Vascular region	Grayish – Green	Grayish – Green
Pith	Grey	Grey
Result	Blue or Blue green	Blue or Blue green
Terpenoids		
Epidermis	Pink	Pink
Hypodermis	Brown – Green	Orange
External cortex	Pink	Pink
Vascular region	Grey – Green	Orange – Green
Pith	Grey	Green – Grey
Result	Orange	Orange

histochemical studies of *B.rubra* stem anatomy of *in vivo* and *in vitro* plants.

The histochemical assay revealed some difference in the phytochemical constitutions between *in vivo* and *in vitro* plants. The presence of alkaloids is higher in *in vivo* plants, especially higher in epidermis hypodermis external cortex and vascular region. Ascorbic acid has shown no difference between *in vivo* and *in vitro* histochemistry. Polyphenols are higher in *in vivo* plant and located in external cortex and vascular region. The concentrations of tannin are identical in *in vivo* and *in vitro* plants. Terpenoids are present in higher concentration in *in vivo* plants especially in hypodermis and vascular regions (Fig-1 and 2). These results are comparable with the previous work of Toshiyuki *et.al* (2001), Ozela *et.al* (2007), Adenegan and Akinnubi (2015), Kumar *et.al* (2015a) and Kumar *et.al* (2015b).

The present work is aimed at the *in vitro* shoot induction studies of *B.rubra.* It has been proved that the surface sterilization of explants is very essential for establishment, pathogen free as well as optimum induction of

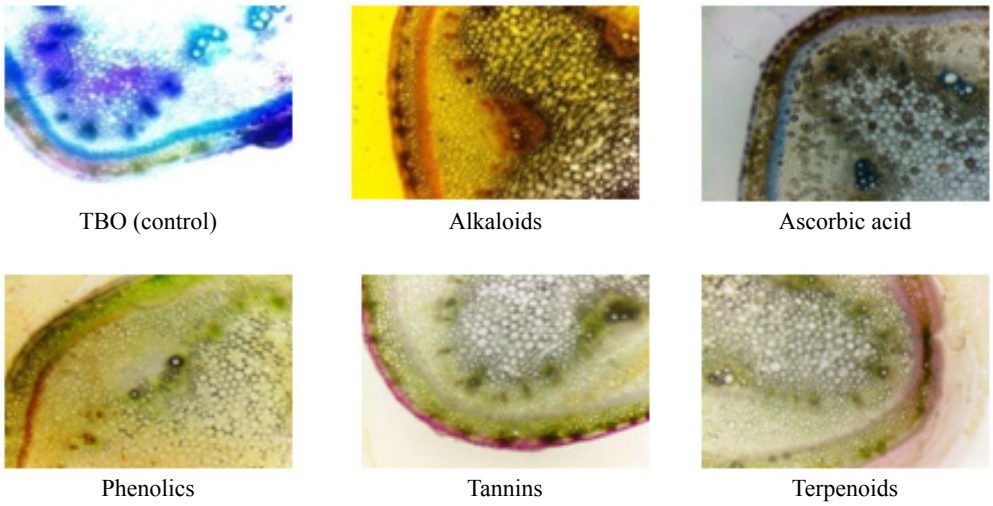


Fig. 1. *In vivo Basella rubra* stem anatomical section used for Histochemical studies

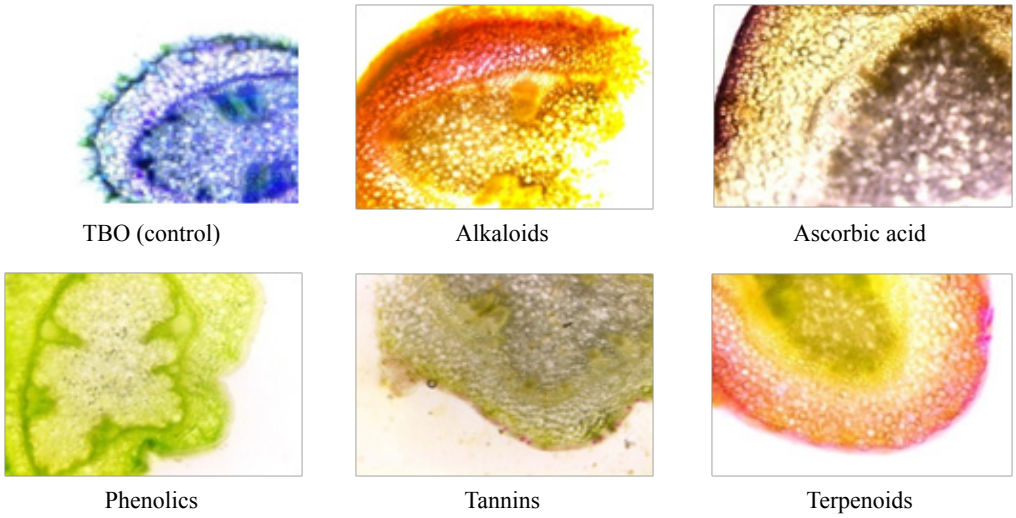


Fig. 2. *In vitro Basella rubra* stem anatomical section used for Histochemical studies

callus and organogenesis *in vitro*. In the present study mercuric chloride was used to sterilize the explants. The appropriate concentration and time for surface sterilization is 1.0% mercuric chloride for 3 minutes for the stem.

Shoot regeneration

In the present study following media concentration and combination were used for root and shoot induction. The following concentration and combination of hormones are used:

MS + IBA 1.0 mg/l + NAA 0.5 mg/l + IAA 1.0 mg/l
MS + IBA 2.0 mg/l + NAA 0.5 mg/l + GA3 0.5 mg/l
MS + IBA 3.0 mg/l + IAA 1.0 mg/l

Shoot induction are initiated in 10 days the other hormones like IAA, NAA have shown but much organogenesis result after inoculation on MS medium supplemented with different concentration and combination of above mentioned hormones and shoot regeneration in 20 days further growth in 30, 40 and 60 days. Among the various combinations, 100% shoot regeneration in concentration of hormones such as MS + BA (1.0 mg/l) produced multiple shoot, in addition to this Kin (0.5, 1.0 mg/l) in the presence of BA induced more number of multiple growths in *Basella rubra*. The present study reveals that MS+BAP (1.0 mg/l), NAA (0.5 mg/l), IAA (1.0 mg/l) as well as MS+BAP (1.0 mg/l), NAA (0.5mg/l) and GA_3 (0.5 mg/l) produced shoot regeneration in *Basella rubra*. The number of shoots produced in this study was on par with earlier reports by Guo and Bin (2001), Pumchaosuan and Wongroung (2009) with reference to *B.alba* and *B.rubra*.

Root Regeneration

In the present study following media concentration and combination were used for root induction:

MS + IBA 1.0 mg/l, MS + BAP 1.0 mg/l
MS + IBA 3.0 mg/l

Root induction are formed in 15 days after inoculation on MS medium supplemented with different concentration and combination of above mentioned hormones and further growth occurred in 30, 40, 60 days (Fig-4 a to d). Among the different combination MS + IBA 1.0 mg/l induced root induction in *B.rubra.* The worked carried out by Guo and Bin (2001), Pumchaosuan and Wongroung (2009), Shekhawat and Manokari 2016 support the present findings.

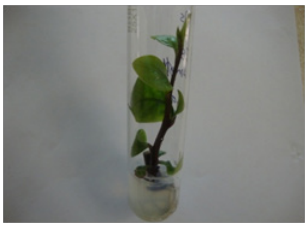
Plantlet regeneration

In the present study following media concentration and combination were used for plant regeneration induction:

a)Green house grown plant

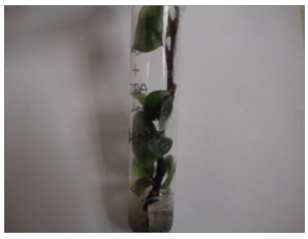
b)Organogenesis in 20 days old culture

c) Organogenesis in 30 days old culture



d) Organogenesis in 30 days old culture (further growth occur)

e) Organogenesis in 60 days old culture



f) Organogenesis in 90 days old culture

Fig. 3. *Basella rubra* stem explants *in vitro* propagation

MS + IBA 0.5 mg/l + GA3 0.5 mg/l + IAA 0.5 mg/l, MS + IBA 0.5 mg/l + GA3 1.0 mg/l + IAA 1.0 mg/l and
MS + IBA 0.5 mg/l + GA3 1.5 mg/l + IAA 1.0 mg/l.

Plant regeneration is maximum in the concentration MS + IBA 0.5mg/l + GA3 1.5 mg/l + IAA 1.0 mg/l (Fig-3) According to Hong *et.al* (2003) propagation of *B.rubra* by stem cutting into MS + BAP 1.0 mg/l + NAA 0.5 mg/l resulted good response the present work reveals that MS + IBA 1.0 mg/l combination has better response to plant regeneration in *B.rubra* These results are comparable with the work of Guo and Bin (2001), Tanikan Pumchaosuan and Sasitorn Wongroung (2009) and Shekhawat and Manokari (2016).

Protocol for *Basella rubra* stem explants *in vitro* propagation:

Ï% The selected stem explants were brought to the laboratory and kept in refrigerator for two days.
Ï% Surface sterilization with 75% alcohol for 50 seconds and 0.1% $HgCl_2$ for 3 minutes.
Ï% Washed thrice in double distilled water.
Ï% Shoot induction with MS + IBA 1.0 mg/l + NAA 0.5 mg/l + IAA 1.0 mg/l.

(Or)

Ï% MS + BAP 1.0 mg/l + NAA 0.5 mg/l + GA3 0.5 mg/l.
Ï% Root induction with MS IBA 1.0 mg/l + IBA 2.0 mg/l.
Ï% Plant regeneration with MS + IBA 0.5 mg/l + GA3 1.5 mg/l +IAA 1.0mg/l
Ï% Incubated in light 1500 Lux at $25^0C \pm 2^0C$.

Nutrient uptake studies by *in vivo* and *in vitro* same plants by SEM- EDX analysis

The SEM-EDX elemental analysis of *in vivo* from *B.rubra* have shown the elements carbon, oxygen, sodium, magnesium, silica, phosphorus, sulphur, chlorine and potassium. The concentration of potassium was 5.82%. The *in vitro* plants have shown carbon, oxygen, magnesium, silica, chlorine, potassium, calcium

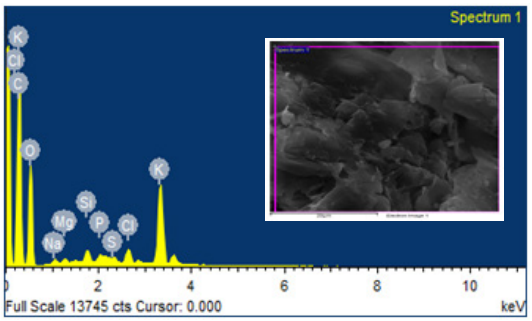


Fig. 4.

SEM – EDX Elemental analysis of *In vivo Basella rubra L.*

Table 2.

S.No	Element	Weight %	Atomic %
1	C K	52.16	61.64
2	O K	39.48	35.02
3	Na K	0.39	0.24
4	Mg K	0.25	0.15
5	Si K	0.58	0.29
6	P K	0.25	0.11
7	S K	0.17	0.08
8	Cl K	0.90	0.36
9	K K	5.82	2.11
10	Totals	100.00	

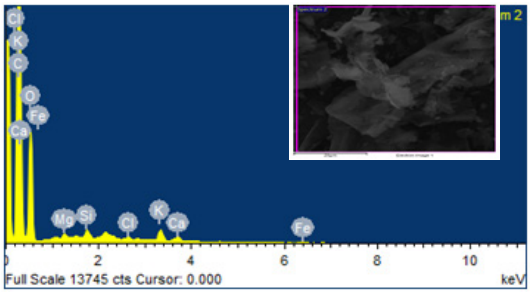


Fig. 5.

SEM – EDX Elemental analysis of *In vitro Basella rubra L.*

Table 2.

S.No	Element	Weight %	Atomic %
1	C K	64.23	71.01
2	O K	34.22	28.40
3	Mg K	0.18	0.10
4	Si K	0.26	0.12
5	Cl K	0.14	0.05
6	K K	0.59	0.20
7	Ca K	0.24	0.08
8	Fe K	0.14	0.03
9	Totals	100.00	

and iron (Table – 2). The result has shown that there is a significance difference in the absorption of nutrients. The *in vivo* plant absorbs sodium, phosphorus and sulphur. Whereas the *in vitro* plants are capable of absorb calcium and iron from the medium (Fig-4). The present study of nutrient uptake by SEM-EDX analyses is a tool to identify selective absorption of minerals by *in vitro* and *in vivo* plants as reported by Abubacker and Sathya (2017).

CONCLUSION

Due to over exploitation of plant source from natural environment for medical uses, the standardization and optimization of shoot regeneration protocol for *B.rubra* because imminent. The present histochemical and shoot induction studies would facilitate the phytochemical constituents and conservation of this important plant species.

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REFERENCES

1. Abubacker M N, and Sathya C. *In vitro* Bioaccumulation of Heavy Metals by Water Lettuce *Pistia stratiotes Engl* (Araceae) and its Combustion Process as Manure Value by SEM-EDX Analysis. *Biolife,* 2017; **5**: 38 – 43.
2. Adenegan TAA and Akinnubi MF. Some anatomical features of *Basella L*. their adaptive significance to water stress. *Res Plant Biol,* 2015; **5**: 14–22.
3. Bamidele O, Akinnuga AM, Olorunfemi JO, Odetola OA, Oparaji CK and Ezeigbo N. Effects of aqueous extract of Basella alba leaves on haematological and biochemical parameters in albino rats. *African Journal of Biotechnology*, 2010; **9**: 6952–6955.
4. Cyunel E. *Basella alba L. In vitro* culture and the production of betalains. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, *Medicinal and aromatic plants II. Springer*, Berlin, New York, 1989; **7**: 47–68.
5. Guo D and Bin X. Tissue culture and plantlet regeneration of *Basella alba L. Acta Agric Zhejiang,* 2001; **13**: 16–18.
6. Kartha KK and Gamborg OL. Elimination of cassava mosaic disease by meristem culture. *Phytopathol*, 1975; **65**: 826–828.
7. Kumar S S, Manoj P and Giridhar P. A method for red-violet pigments extraction from fruits of Malabar spinach (*Basella rubra*) with enhanced anti-oxidant potential under fermentation. *Journal of Food Science and Technology*, 2015a; **52**: 3037-3043.
8. Kumar S S, Manoj P, Giridhar P, Shrivastava R and Bharadwaj M. Fruit extracts of *Basella rubra* that are rich in bioactives and betalains exhibit antioxidant activity and cytotoxicity against human cervical carcinoma cells. *Journal of Functional Food*, 2015b; **15:** 509-515.
9. Larkcom J. *Oriental vegetables: the complete guide for garden and kitchen*. Kodansha International, USA. 1991.
10. Maisuthisakul P, Ritthiruangdej PS. Relationship between antioxidant properties and chemical composition of some Thai plants. *J Food Compost Anal,* 2008; **21**: 229–240.
11. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant*, 1962; **15**: 473–497.
12. Mundo L, Gorospe KJ, Lorilla L, Serquina AK and Torres E. The Feasibility of *Basella rubra L*. as a biological stain. *Bato Balani Sophomore*, 1995; **14**: 16–18.
13. Ozela EF, Stringhet PC and Chauca MC. Stability of anthocyanin in spinach vine (*Basella rubra*) fruits. *Cien Investig Agrar*, 2007; **34**: 115–120.
14. Sankar NR, Sreeramulu A, Gopal DS and Bagyanarayana G. First report of leaf blight of *Basella alba* caused by Alternaria alternata in India. *Plant Dis,* 2011; **95** - 1476.
15. Shekhawat MS and Manokari M. Optimization of *in vitro* and *ex vitro* regeneration and micromorphological studies in *Basella alba L. Physiol Mol Biol Plants*, 2016; 123:**22**: (4) 605–612.
16. Shanmugam S, Sathish Kumar T and Panneer Selvam K. *Laboratory Handbook On Biochemistry* PHI learning private ltd, New Delhi, 2010.
17. Tanikan Pumchaosuan and Sasitorn Wongroung. In vitro propagation of ceylon spinach (Basella

rubra L.). *As. J. Food Ag-Ind*, 2009; S31-S36.

18. Toshiyuki M, Kazuhiro H and Masayuki Y,. Structures of new oleanane-type triterpene oligoglycosides, *Basella* saponins A, B, C, and D, from the fresh aerial parts of *Basella rubra L*. *Chemical and Pharmaceutical Bulletin*, 2001; **49:** 776 - 779.

19. Yang RY, Lin S and Kuo G. Content and distribution of flavonoids among 91 edible plant species. *Asia Pac J Clin Nutr*, 2008; **17**: 275–279.