Pak. J. Bot., 45(1): 197-202, 2013.

STANDARDIZATION OF TISSUE CULTURE CONDITIONS AND ESTIMATION OF FREE SCAVENGING ACTIVITY IN *VIOLA ODORATA* L.

MUHAMMAD NAEEM[1], ISHRAT NAVEED[2], SYED MUHAMMAD SAQLAN NAQVI[3] AND TARIQ MAHMOOD[1*]

[1]*Department of Plant Sciences, Quaid-i-Azam University, Islamabad-45320, Pakistan*
[2]*Department of Biotechnology, Quaid-i-Azam University, Islamabad-45320, Pakistan*
[3]*Department of Biochemistry, PMAS Arid Agriculture University Rawalpindi, Pakistan*
**Corresponding author's e-mail: tmahmood@qau.edu.pk; tmahmood.qau@gmail.com*

Abstract

Tissue culture can be used as a tool for the conservation and commercialization of *Viola odorata* because of its intensive use in local market for medicinal purposes. In the present study an efficient protocol for *in vitro* callogenesis and organogenesis of medicinally important plant *Viola odorata* L. (Sweet violet) has been developed. Different explants used for callus induction were leaves, stem and petioles. Best callus induction (85%) was observed on media having 6-benzylaminopurine (BA) 2.5 mg/L and 2, 4-dichlorophenoxyacetic acid (2, 4-D) 0.15mg/L after 40 days of incubation. Subsequent transfer of callus to shooting media has shown best shoot regeneration (having 4-5 cm length and 2-3 branches) on medium supplemented with 1-naphthaleneacetic acid (NAA) 0.5 mg/L, gibberellic acid (GA_3) 1.5 mg/L, $AgNO_3$ 0.42 mg/L and thidiazuron (TDZ) 2.5 mg/L. An assay of the antioxidant potential of the *in vitro* grown callus and the wild plant extract was determined by DPPH (α, α-diphenyl-β-picrylhydrazyl) method shown that the antioxidant activity of *in vitro* formed callus is higher than that of wild plant.

Introduction

Time immemorial phytochemicals are being used for health care especially in underdeveloped countries and remote areas (Gul *et al*., 2012). Nearly 30% of drugs across the globe are derived from plants and 252 drugs are in WHO essential medicine list (Sahoo *et al.,* 2010). *Viola odorata* commonly known as sweet violet or garden violet belongs to family Violaceae, is a low growing perennial, with stout root stock, grows in hedgerows, rough land and margins of woodland (Tobyn *et al.,* 2011), *V. odorata* is considered to be a native plant of Southern Europe, North Western Africa and Western Asia. In Pakistan it is found in Southern hilly regions of Swat, Hazara, Muree and Kashmir. The viola is rich in secondary metabolites including, flavonoids, alkaloid (violin, v*iola*-quercitin), essential oils including (ionones, alpha-ionone, beta-ionone and beta-dihydroionone, hydroquinone dimethyl ether, linolenic) and extensively used in diuretic, anti-inflammatory, purogative properties, abdominal pain, skin disorders, upper respiratory complications (cough, sore throat and harash) (Kathi, 1991; Lamaison *et al.,* 1991; Hansel *et al.,* 1993; Svangard *et al.,* 2004; Jackson & Bergeron, 2005; Witkowska -Banaszczak *et al.,* 2005, Walter *et al*., 2011).

Antioxidants are substances, usually of plant origin that reduce and neutralize free radicals and play a vital role in the prevention of cancer, cardiovascular diseases and neurodegenerative diseases including Alzheimer and Parkinson diseases (Gerber *et al.,* 2002; Di & Esposito, 2003). In *Viola odorata* antioxidant activity is related to the amount of anthocyanins, one of the groups of flavonoids pigments. Anthocyanins occur in all tissues including leaves, stems, roots and flowers. Antioxidants like phenolic acids, polyphenols and flavonoids etc also show their effect by scavenging free radicals, preventing the generation of reactive oxygen species (ROS) or activating detoxifying proteins (Halliwell *et al.,* 1992; Halliwell *et al.,* 1999). Natural antioxidants are widely used and regarded as safer and preferable having less adverse reaction as compared to synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are suspected to have some toxic effects (Imaida *et al.,* 1983). However, one drawback of natural antioxidants is that it has lower antioxidant activities than synthetic antioxidants. Therefore, there is a great concern in finding safe and new efficient antioxidants from natural sources to replace these synthetic antioxidants (Gazzani *et al.,* 1998).

Safe use of medicinal plants led to sudden rise in market demand that results over-exploitation and ultimate decline in natural habitat. Medicinal plants all across the globe getting endangered because of their ruinous harvesting for the production of medicines as most of them grown as a wild under natural conditions. The germplasm of *Viola odorata* conserved in natural repositories is under severe intimidation because of its intensive use in local market for medicinal purposes and not being cultivated commercially to fulfill its markets requirements and currently rare in Pakistan. *In vitro* propagation methods deliver powerful methods for the mass multiplication and germplasm conservation of economically important species (Snyman *et al.,* 2000). Previously Babber & Kulbhushan (1991) in an experiment got callus from root hypocotyls and cotyledonary segments of *Viola tricolor* but unsuccessful to get shoot formation. In a report Sato *et al.,* (1995) managed regeneration of plantlets from petiole callus of wild *Viola*. In yet another report Wijowska *et al.,* (1999) achieved callus independent endosperm and root *In vitro* by culturing unfertilized ovules of *Viola odorata*. There are various reports about the breeding, inheritance, cultivation and regeneration of *Viola odorata* (Rupinder & Ramash, 1998), however to date there have been no reports on the successful *In vitro* callogenesis and organogenesis of *Viola odorata* using leaf explant. The present study has been carried out to establish an efficient *In vitro* protocol of callogenesis and organogenesis for *Viola odorata* by optimizing the various combinations of hormones and to evaluate and compare antioxidant activity of wild plant and its derived callus by DPPH method.

Material and Methods

Sterilization and inoculation of explants: Leaves, stem and petioles were collected from ~45 days older plants of *Viola odorata* maintained in the green house. The explants were washed with distilled water to remove the dust and soil particles and then were kept in 70% ethanol for one minute and in 50% Clorox (bleach) for 8-10 minutes. The bleach was removed by washing thrice with autoclaved distilled water under aseptic conditions. The explants were kept on sterile filter paper (Wattman 1) in a petri plate for drying. The leaves, stem and petiole were cut into small pieces of 10-15mm in size rectangular or square in shape with the help of a sterilized surgical blade and were inoculated on half strength MS basal medium (the adaxial side was in contact with media).

Media preparation: For callus culture of *Viola odorata* half and full strength MS media (with 15g/L and 30g/L sucrose respectively) were used supplemented with twenty different concentrations and combinations of hormones (Table 1). The pH of media was adjusted at 5.8 and 2 g/l gelling gum powder was used to solidify the media.

Table 1. Media with different combinations of hormones used for callus induction of *Viola odorata*.

S. No.	Media code (C)*	Half (H)/Full (F) MS	PGR's	Concentration (mg/L)
1.	C1	H	BA+2, 4-D	2+0.11
2.	C2	H	BA+2, 4-D	2.2+0.12
3.	C3	H	BA+2, 4-D	2.3+0.13
4.	C4	H	BA+2, 4-D	2.5+0.15
5.	C5	H	BA+2, 4-D	2+1.9
6.	C6	H	BA+2, 4-D+NAA	2.5+0.1+0.15
7.	C7	F	BA+2, 4-D	2+0.11
8.	C8	F	BA+2, 4-D	2.3+0.13
9.	C9	F	BA+2, 4-D	2.5+0.15
10.	C10	H	BA+2,4-D+Kin	1.5+1+0.1
11.	C11	H	BA+2,4-D+Kin	0.5+1+0.4
12.	C12	H	BA+IAA+Kin	1.5+1+.0.1
13.	C13	H	BA+IAA+Kin	3+2+0.4
14.	C14	H	BA+NAA	2.2+0.54
15.	C15	H	Kin+NAA	2+0.8
16.	C16	F	Kin+2,4 D	2+0.15
17.	C17	F	Kin+IAA	1.5+0.2
18.	C18	H	BA+2, 4-D	4+0.22
19.	C19	H	BA+2, 4-D	4.4+0.26
20.	C20	H	BA+2, 4-D	5+0.3

C*: Callus induction media

Culture conditions: The callus culturing was done on media present in petri plates. These cultures were kept in 16 hrs photoperiod, 55±5% relative humidity at 25±1°C and the light intensity was maintained at 1000 lux inside growth chamber for callus induction. Color, size and texture of callus were monitored at regularly intervals. All the parameters were monitored by comparative examination.

Shoot formation: After successful callus induction it was transferred to another half strength MS media called shooting media supplemented with different combinations and concentration of Thidiazuron (TDZ), Silver nitrate ($AgNO_3$), gibberellic acid (GA_3) and 1-naphthaleneacetic acid (NAA) (Table 2). The shooting media were poured in 250ml glass bottles inside the laminar flow hood. After inoculation of callus on shooting media the bottles were kept inside the growth chamber for shoot initiation under the same conditions as adjusted for callus induction.

Antioxidant activity: The capability of prepared extracts to scavenge free radical DPPH (α, α-diphenyl-β-picrylhydrazyl) was then determined by the method of Amarowicz *et al.,* (2004).

Results

Callogenesis: Callus induction was observed on 9 media combinations out of the total 20 media combinations used for callus induction. Significant difference like color, size, time of callus induction was recorded (Table 3) along with the percentage callus induction (total callus/total number of disc) formed on these media (Fig. 2). C1 and C2 media induced 50-55% callus (Fig. 2) after 65 days

having BA (2 and 2.2 mg/L, respectively) and 2, 4-D (0.11 and 0.12 mg/L) and the callus was light green, small and compact (Table 3). C3 medium having BA (2.3 mg/L) and 2, 4-D (0.13 mg/L) induced medium sized, compact and light green colored callus with up to 70% after 55 days. Best and efficient callus induction occurred on C4 medium which was up to 85% (Fig. 2) after 40 days of incubation having BA (2.5 mg/L) and 2, 4-D (0.15 mg/L) and the callus was observed to be dark green, compact and larger in size (Table 3).

Table 2. Shooting media with different hormonal combinations.

Media code (S)*	PGRs	Concentration (mg/L)
S1	NAA+GA_3 +$AgNO_3$ +TDZ	0.2+0.99+0.4+2
S2	NAA+GA_3 +$AgNO_3$ +TDZ	0.5+1.05+0.42+2.5
S3	GA_3 +$AgNO_3$ +TDZ	1.2+0.45+1
S4	NAA+GA_3+$AgNO_3$	0.5+1.5+ 0.2

S*: Shooting media

Table 3. Data of callus induction on MS media with different combinations of hormones.

S. #	Media code	Half(H)/Full(F) MS media	PGRs	Concentration (mg/L)	% age	Colour	Texture	Size	Days
1.	C1	H	BA+2, 4-D	2+0.11	50	Light green	Compact	Small	65
2.	C2	H	BA+2, 4-D	2.2+0.12	55	Light green	Compact	Small	65
3.	C3	H	BA+2, 4-D	2.3+0.13	70	Light green	Compact	Medium	55
4.	C4	H	BA+2, 4-D	2.5+0.15	85	Dark green	Compact	Large	40-45
5.	C8	F	BA+2, 4-D	2.3+0.13	25	Light brown	Crystalline	Small	70
6.	C9	F	BA+2, 4-D	2.5+0.15	30	Whitish brown	Crystalline	Medium	65-70
7.	C10	H	BA+2, 4-D+Kin	1.5+1+0.1	60	Greenish brown	Granulated	Large	50-60
8.	C11	H	BA+2, 4-D+Kin	0.5+1+0.4	20	Yellow	Granulated	Small	70
9.	C12	H	BA+IAA+Kin	1.5+1+.0.1	25	Greenish brown	Granulated	Medium	60-65

Callus induced on full strength MS media i.e., C8 and C9 was not much effective and callus induction was observed after 65-70 days having crystalline, whitish brown and medium sized callus. Among C10 and C11 (both on half strength MS media) best callus induction occurred on C10 up to 60% having BA 1.5 mg/L, 2, 4-D 1 mg/L and kinetin 0.1 mg/L and the callus was granulated, greenish brown and large in size. C12 medium (half strength MS media) containing IAA 1 mg/L instead of 2, 4-D along with BA 1.5 mg/L and Kinetin, 0.1 mg/L induced 25% granulated medium sized callus having greenish brown color after 60-65 days of incubation (Fig. 1a).

Response of explants on callogenesis: The potential of callus induction varied among different parts of *V. odorata* used as an explant i.e., leaves, stem and petioles. Callus induction potential was found higher in leaves as compared to stem and petioles in which callus was found throughout the leaf disc while in stem and petiole, callus was localized to the tips and terminal parts. Moreover, it was observed that young and green explants were efficient in callus formation than older ones, diseased and spotted explants. It was observed that callus from stem and petiole became decolorized after few days of incubation (Fig. 1c).

Shooting: In total four different media (S1 to S4) were used for regeneration. S1 medium having NAA 0.2 mg/L, GA_3 0.99 mg/L, $AgNO_3$ 0.4 mg/L and TDZ 2 mg/L induced 65% shooting after 65 days with 1-2 branches having 2-3 cm length . Best shooting occurred in S2 medium having NAA 0.5 mg/L, GA_3 1.05 mg/L, $AgNO_3$ 0.42 mg/L and TDZ 2.5 mg/L initiating 80% shooting after 30 days having 2-3 branches and 4-5 cm height (Fig. 1d, 1e and 1f). No or very slow shooting occurred in media not supplied with $AgNO_3$ or TDZ. Another shooting medium, S3 with NAA 0.3 mg/L, GA_3 1.2 mg/L and TDZ 1.5 mg/L without $AgNO_3$ induced only 25% after 45 days of incubation with only one branch of 2 cm length showing the role of $AgNO_3$ in regeneration. S4 medium NAA (0.5mg/L), GA_3 (1.5mg/L) and $AgNO_3$ (0.2mg/L) devoid of TDZ induced 15% shooting after 50 days having 2 cm long weak shoot. In general it was observed that the green, compact and larger calli have shown rapid shooting on shooting media as compared to small sized whitish or brownish calluses.

Antioxidant activity: Antioxidant activity was evaluated by DPPH method. It was observed from the data obtained that *In vitro* formed callus had higher antioxidant activity 38% while that of wild plant extract was 33%. The antioxidant activity of wild plant is 5% less than its derived callus.

Fig. 1. Callus induction and shooting of *Viola odorata*; (a) callus from leaf with green color; (b) callus with greenish brown color; (c) callus from stem and petiole; (d) callus derived shooting with single branch; (e) plantlet with two branches; (f) plantlet with more branches.

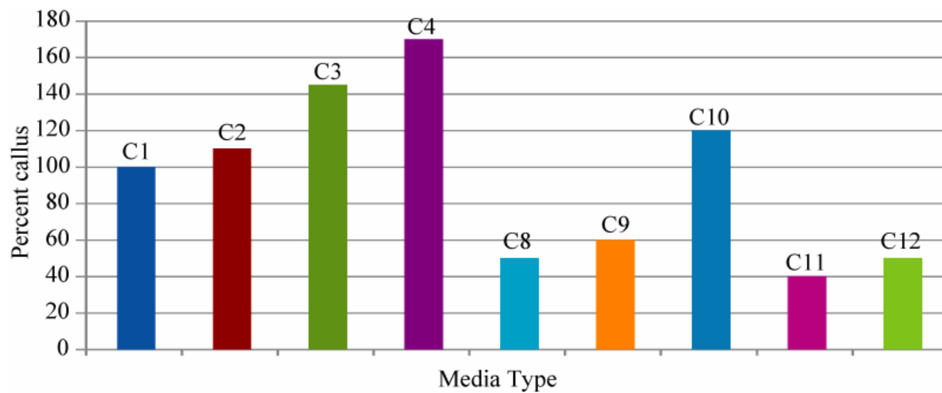

Fig. 2. Percentage callus induction of *Viola odorata* (total callus/total number of disc) on media having different hormonal combinations. C1: (100/200) 50%; C2: (110/200) 55%; C3: (145/200) 70%; C4: (170/200) 85%; C8: (50/200) 25%; C9: (60/200) 30%; C10: (120/200) 60%; C11: (40/200) 20%; C12: (50/200) 25%.

Discussion

In the present study, the effect of various plant growth regulators such as BA (Benzyl aminepurine), 2, 4-D (dicholorophenoxy acetic acid), IAA (Indolacetic acid) Kinetin and NAA (Naphthalene acetic acid) on callogenesis and organogenesis was determined and an efficient protocol for tissue culture of *Viola* was established. Among all these hormones BA and 2, 4-D with varying concentration induced callus in media C1-C4 sin which best and efficient callus formed in media C4 (Table 3). Previously Wijowska *et al*., (1999) achieved callus by *In vitro* culturing unfertilized ovules of *Viola odorata* using these hormones.

The amount and concentration of micro and macro nutrients of MS media is also important for callus induction. Efficient callus induction was observed when half strength MS media was used as compared to full strength MS having same hormonal concentration. This result is an agreement with the available reports for *Viola tricolor* (Wang & Man, 2006) and *Hybanthus floribundus* (Bidwell *at al.,* 2001) in which callus induction was optimized on half MS media. However in case of ovule callus induction for *Viola odorata* (Wijowska *et al.,* 1999) and *Hybanthus enneaspermus* belonging to Violaceae, full strength MS media was optimized for callus induction and it was found to be more suitable (Parkash *et al.,* 1999).

Best shooting occurred on media having TDZ and $AgNO_3$ along with plant hormones GA_3 and NAA. This combination was used from the report of Wang & Man (2006), when they used the same combination for efficient shooting in case of *Viola wttirockinii.* The Shooting efficiency, height and number of branches seems to be dependent upon the concentration of TDZ and $AgNO_3$ along with other hormones (Table 4). TDZ is known to be more active than zeatin for stimulating the growth when added to a tissue culture medium at low concentration (Sajid & Faheem 2009). In another report it has been considered to be more potent than most of the commonly used cytokinins (Huetteman & Preece, 1993). $AgNO_3$ is also known to promote multiple shoot formation in different plants. Moreover, *In vitro* shoot formation was improved by incorporating $AgNO_3$ in the culture medium. Earlier in a similar study, Ganesh & Sreenath (1996) reported *In vitro* sprouting of apical buds of *coffea* under the influence of $AgNO_3$.

Table 4. Data of shooting on MS media containing different combinations of hormones.

Media code	PGRs	Concentration (mg/L)	Percent shooting	Days	Length (cm)	No. of shoots
S1	$NAA+GA_3+AgNO_3+TDZ$	0.2+0.99+0.4+2	65	35-40	2-3	1-2
S2	$NAA+GA_3+AgNO_3+TDZ$	0.5+1.05+0.42+2.5	80	30	4-5	2-3
S3	$NAA+GA_3+TDZ$	0.3+1.2+1.5	25	45	2	1
S4	$NAA+GA_3+AgNO_3$	0.5+1.5+ 0.2	15	50	2	1

Presently, it has been observed that the shooting was poor in media devoid of both or one of the chemicals i-e $AgNO_3$ and TDZ. However apart from this, shooting efficiency also depends on callus color, age and type of explant from which callus derived (Orlikowska *et al.,* 1999). It has been observed from the data that green callus have greater organogenic potential in comparison with other calluses. Similar results have been reported in some other plants (Maureen & Pau, 1990).

Antioxidant activity was also monitored in wild plants and from callus of *Viola odorata* with the help of DPPH method previously used by Vukics (2008). It was observed that the callus derived extract have higher antioxidant activity than wild plants derived extract. According to Shilpa *et al*., (2010) *in vitro* callus confirmed enhanced secondary metabolite than explant plant in case of *Viola odorata*. In another report, Abbasi *et al.,* (2010) reported similar results, according to which *Silybum* had a higher antioxidant activity than parent plant. Earlier reports suggested that the phenolic compounds mainly contribute to the antioxidant capacity of 112 wild grown Chinese herbs (Cai *et al.,* 2004).

In case of *Viola odorata*, antioxidant activity is related to the amount of anthocyanins, one of the groups of flavonoids pigment. The total phenol and flavonoids contents in the leaf of *Viola odorata* were determined recently by Ebrahimzadeh *et al.,* (2010) suggesting that the leaves of *Viola odorata* had higher antioxidant activity than other parts of the plants.

Conclusion

Viola odorata is an important medicinal plant and it is grown wild under natural conditions and is over-exploited for the treatment of various ailments. Due to its extensive use in herbal formulation without commercial cultivation became rare and if comprehensive measures would not be taken the *V. odorata* would be under threat. The current study established the protocol for its callus induction and shoot formation for conservation purpose and utilization in pharmacological industry in future. The plant is rich in secondary metabolites and production is limited. Antioxidant activity has been evaluated and compared between wild type of *Viola odorata* and callus. The study found that callus has shown enhanced production of antioxidants and further study need to be carried out recover antioxidant properly.

Acknowledgement

We are thankful to National Agricultural Research Centre (NARC) Pakistan for providing plant material.

References

Abbasi, B.H., M.A. Khan, T. Mahmood, M. Ahmad, M.F. Chaudhary and M.A. Khan. 2010. Shoot regeneration and free-radical scavenging activity in *Silybum marianum* L. *Plant Cell Tissue Org. Cult.,* 101: 371-376.

Amarowicz, R., R.B. Pegg, P. Rahimi-Moghaddam, B. Barl and J.A. Weil. 2004. Free-radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies. *Food Chem*., 84: 551-562.

Babber, S and S. Kulbhushan. 1991. Study of anatomy of vitrified structure in *Viola tricolor* L. *Ann. Biol. (Ludhiana),* 7(1): 93-95.

Bidwell, S.D., J.W. Pederick, K. Sommer and I.E. Woodrow. 2001. Micropropagation of the Nickel hyperaccumalator, *Hybanthus floribundus* (family-Violaceae). *Plant Cell Tiss. & Org. Cult.,* 67(1): 89-92.

Cai, Y.Z., Q. Luo, M. Sun and H. Corke. 2004. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associate with anticancer. *Life Sci.,* 74(17): 2157-2184.

Di, M.V. and E. Esposito. 2003. Biochemical and therapeutic effects of antioxidants in the treatment of Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis. *Current Drug Target CNS and Neurological Disorder,* 2: 95-107.

Ebrahimzadeh, M.A., S.F. Nabavi, S.M. Nabavi and B. Eslami. 2010. Antihypoxic and antioxidant activity of *Hibiscus* esculentus seeds. *Grasas Aceites.,* 61(1): 30-36.

Ganesh, S.D. and H.L. Sreenath. 1996. Silver nitrate enhanced shoot development in cultured apical shoot buds of *Coffea arabica* Cv Cauvery (S4347). *J. Plantation Crop.,* 24: 577-580.

Gazzani, G., A. Papetti, G. Masoolini and M. Daglia. 1998. Anti- and prooxidant activity of water components of some common diet vegetables and the effect of thermal treatment. *Journal Agri. & Food Chem.,* 6: 4118-4122.

Gerber, M., M.C. Boutron, S. Hercberg, E. Riboli, A. Scalbert and M.H. Siess. 2002. Food and cancer. State of the art about the protective effect of fruits and vegetables. *Bull. Cancer,* 89: 293-312.

Gul, F., Z. K. Shinwari and I. Afzal. 2012. Screening of Indigenous Knowledge of Herbal Remedies for Skin Diseases among Local Communities of North West Punjab. *Pak. J. Bot*., 44(5): 1609-1616.

Halliwell, B. and J.M.C. Gutteridge. 1999. *Free radicals in biology and medicine.* 2nd edition Oxford United Kingdom: Clarendon press.

Halliwell, B., J.M.C. Gutteridge and C.E. Cross. 1992. Free radicals, antioxidant and human disease: where are we now? *J. Lab. Clin. Med.,* 119-598.

Hansel, R., R. Keller, H. Rimpler and G. Schneider. 1993. Hagers Handbuch der Pharmazeutischen Praxis. Berlin, Springer-Verlag., 11: 41-53.

Huetteman, A. and E.J. Preece. 1993. Thidiazuron: a potent cytokinin for woody plant tissue culture. *Plant Cell. Tiss. Org. Cult.,* 33: 105-119.

Imaida, K., S. Fukishima, T. Shirai, M. Ohtami, K. Nakamish and N. Ito. 1983. Promoting activities of butylated hydroxyanisole and butylated hydroxytoluene on 2-stage urinary carcinogenesis and inhibition of gamma-glutamyl trans peptidepositive for development in the liver of rats. *Carcinogenesis*, 4: 895-899.

Jackson, D. and K. Bergeron. 2005. *Alternative nature online herbal*. Edited by K. Bergeron http:// www.altnature.com/gallery/violet.htm.

Kathi, K. 1991. *The illustrated herb encyclopedia (a complete culinary, cosmetic, medicinal and ornamental guide to herbs)*. Mallard Press, New York. 207.

Lamaison, J.L., F.C. Petitjean and A. Carnat. 1991. The violet flower, a comparison between *Viola lutea, Viola calerata* and *Viola odorata*. *Plants Med. Phytothera*., 25: 79-88.

Maureen, M. and H.M. Pau. 1990. Comparison of 2, 4-D and picloram for selection of long-term totipotent green callus cultures of sugarcane. *Plant Cell Tiss. Org. Cult.,* 20(3): 157-163.

Orlikowska, T., E. Nowak, A. Marasek and D. Kuchrska. 1999. Effect of growth regulators and incubation period on in vitro regeneration of adventitious shoots from gerbera petioles. *Plant Cell Tiss. Org. Cult*., 59: 95-102.

Parkash, E., P.S. Sha, P. Vallikhan, R. Sairam and K.R. Rao. 1999. Regeneration of plants from seed-derived callus of *Hybanthus enneaspermus* (L.) Muell, a rare ethnbotanical herb. *J. Plant Cell Rep*., 18: 873-878.

Rupinder, K. and K. Ramesh. 1998. Effect of nitrogen, phosphorus and plant spacing on seed yield in pansy (*Viola tricolor*) cv 'Swiss Giant'. *J. Ornamental Hort. New Series.,* 11: 21-25.

Sahoo, N., P. Manchikanti and S. Dey. 2010. Herbal drugs: Standards and regulation. *Fitoterapia.,* 81(6): 462-471

Sajid, Z.A. and A. Faheem. 2009. Effect of Thidiazuron (TDZ) on *in vitro* micropropegation of *Solanum tuberosum* L. CVS. Desiree and cardinal. *Pak. J. Bot.,* 41(4): 1811-1815.

Sato, T., O. Kwon, H. Miyake, T. Tangiguchi and E. Maeda. 1995. Regeneration of plantlets from petiole callus of wild *Viola* (*Viola patrinii* DC.). *Plant Cell Rep.,* 14: 768-772.

Shilpa, K., C. Selvakkumar, A.K. Senthil and B.S. Lakshmi. 2010. *In vitro* root culture of *Ocimum sanctum* L. and evaluation of its free radical scavenging activity. *Plant Cell Tiss. Org. Cult.,* 101: 105-109.

Snyman, S.J., M.P. Watt, F.C. Huckett and B.I. Botha. 2000. Direct somatic embryogenesis for rapid, cost-effective production of transgenic sugarcane (*Saccharum* spp. hybrids). *Pro. South Africa. Sugar Technol. Assoc*., 74: 186-187.

Svangard, E., U. Goransson, Z. Hocaoglu, J. Gullbo and R. Larsson. 2004. Cytotoxic cyclotides from *Viola tricolor*. *J. Nat. Prod.,* 67:144-147.

Tobyn, G., A. Denham and M. Whitelegg. 2011. *Viola odorata*, sweet violet; *Viola tricolor*, heartease. *Medical Herbs.* Chapter 32:337-348.

Vukics, V., T. Ringer, A. Kery, G.K. Bonn and A. Guttman. 2008. Analysis of heartsease (*Viola tricolor* L.) flavonoid glycosides by micro-liquid chromatography coupled to multistage mass spectrometry. *J. Chromatogr. A.,* 1206(1): 11-20.

Walter C., Z. K. Shinwari, I. Afzal and R.N. Malik. 2011. Antibacterial Activity in Herbal Products Used in Pakistan. *Pak. J. Bot*., 43 (Special Issue): 155-162.

Wang, J. and Z.B. Man. 2006. Plant regeneration of pansy (*Viola wittrockiana*) Caidie via petiole-derived callus. *Scientia Horticult.,* 111: 266-270.

Wijowska, M., E. Kuta and L. Przywara. 1999. *In vitro* culture of unfertilized ovules of *Viola odorata* L. *Acta Biol Cracov Ser. Bot.,* 17: 1-11.

Witkowska-Banaszczak, E., W. Byka, I. Matlawska, O. Goslinska and Z. Muszynski. 2005. Antimicrobial activity of *Viola tricolor* herb. *Fitoterapia*, 76: 458-461.

(Received for publication 1 August 2011)