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IN VITRO MICROPROPAGATION OF *SOLANUM VILLOSUM*–A POTENTIAL ALTERNATIVE FOOD PLANT

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Abstract

Solanum villosum Miller is annual to biennial herb which is used as potherb as well as fodder/forage that limits its distribution in Pakistan. The aim of this study was to develop a suitable protocol for *S. villosum* through direct organogenesis. Leaf, stem node and shoot tip explants from the tested plant were inoculated in three different hormonal combinations of BAP (6-benzyl amino purine) alone along NAA (α-naphthalene acetic acid) and Kin (Kinetin). Maximum shoot induction was recorded for stem node and leaf (91% each) in MS medium comprising of BAP (1.9 mg/l) and NAA (0.1 mg/l), while shoot tip showed somewhat moderate (81%) response. The highest mean number of shoot (9.1±0.12) was also obtained for the same medium using leaf explants. Plantlets were successfully rooted in auxin free medium and shifted to green house for multiplication after acclimatizing them. This study may contribute in providing quick and disease free propagation of this neutraceutically and economically potential plant.

Key words: Micropropagation, Explants, *Solanum villosum*, Direct regeneration, MS medium, Plantlets.

Introduction

Solanum villosum Miller, commonly known as red-fruit nightshade (locally as *Kaach Maach*) is a medicinal herb. It is an annual to biennial plant up to 50 cm high. Berries are usually longer than wide, seldom globose, red, orange or yellow with1.6-2.2 mm long seeds (Edmond & Chweya, 1997). It is distributed in different areas of Pakistan such as Kotli, Poonch, Multan, D. I. Khan, Quetta, Rawalpindi, Attock, Jhang, Lahore, Hazara, Bannu, Gilgit and Sibi (Yousaf *et al.*, 2010).

S. villosum being an ayurvedic herb, is reported to have multiple medicinal properties. Its ground and soaked leaves has been reported to remove swellings, sore eyes, marasmus' or 'kwashiokor'. Besides, its leaves are also eaten as boiled salad and orange berries are used as fruit and are also recommended for pregnant women for the birth of children with dark eyes and even skin, moreover for ease in recuperation after delivery (Edmond & Chweya, 1997). *S. villosum* is also used to make jams and are well suited for pies, preserves and as a substitute for raisins in plum puddings, particularly in North America (Fisher, 1977). Several neutraceutical investigations revealed that the leaves are blessed with protein and methionine; an amino acid limited in other vegetables, calcium, iron, phosphorus, vitamins A, B and C, fat and fiber, carotene (Fortuin & Omta, 1980). Although this species is known as troublesome weed across the world, however in India and Pakistan, this species is not the weeds of momentous agronomic value, except under irrigation in localized areas instead it has been recorded as being used as a trifling crop (Schilling & Andersen, 1990).

Medicinal plants are the most important source of life saving drugs for the majority of the world's population. Also the biotechnological methods are important to select, multiply and conserve the critical genotypes of medicinal plants. *In vitro* regeneration holds tremendous potential for the production of high quality plant-based medicine (Pandhure *et al.,* 2010). The term *In vitro* cultures, micro-propagation or regeneration covers a broad range of techniques involving the escalation under germ-free condition of plant germplasm (especially shoot tips, meristem, somatic embryos or embryonic callus) on non-natural culture media and can produce thousands or even millions of plants (Ugandhar *et al.*, 2012). The victory of the micropropagation depends on plentiful factors like genotype, medium, plant growth regulators and category of explants, which should be pragmatic during the process (Kim *et al.*, 2003).

Several studies reported micropropagation of many leafy vegetables of Solanaceae such as *S. lycopersicum* (Chaudhry *et al.*, 2010), *S. melongena* (Magioli & Mansur, 2005; Ray *et al.,* 2011) and S. *nigrum* (Padmapriya *et al.*, 2011; Kolar *et al.*, 2008). Efforts have been started recently to micro propagate *S. villosum* due to its emerging importance in developing countries as ethnomedicinal, vegetable and fruit plant (Hussein, 2011; Hussein & Aklan, 2011). But still all possible types of explants and hormonal combinations have not been exploited to develop more diverse and efficient protocols. Keeping in view, present study had been planned to establish micro-propagation protocol *via* direct organogenesis using stem node, leaf and shoot tip explants of *S. villosum*.

Materials and Methods

The present research work was carried out at PTC lab of Botany Department, PMAS Arid Agriculture University Rawalpindi Pakistan during 2013-2014.

Identification and collection of plant material: Plant specimens along with fresh material for tissue culture were collected from wild and unattended patches of Arid Agriculture University Rawalpindi Pakistan. One set of specimen was pressed, dried, mounted and identified with the help of floristic literature (Zhang *et al.*, 1978). The voucher specimen was deposited in the Department of Botany, PMAS Arid Agriculture University Rawalpindi. Young as well as mature but buds possessing, apparently

disease free plants along with their roots were dug out and transferred to pots to green house of Botany Department for future use. For SAM explants seeds were harvested and Plantlets of 3-4 weeks old served as a healthy source for shoot tip explants.

Explant collection and sterilization: Young leaf segments (horizontally cut from basal portion near petiole) and stem nodes were randomly cut from *S. villosum* while shoot tips were cut from 4 week old plantlets. All were then sterilized with modified standard protocol of Ragavendran *et al.* (2014). Explants were initially washed with tap water. Afterwards rinsed with autoclaved distilled water with 1-2 drops of tween 20 followed by 20% chlorax solution having 1% sodium hypochlorite, side by side washing with autoclaved distilled water to remove chemicals properly. Beakers having explants were covered with Al foil and were kept in sterile filter paper for drying before inoculation.

Culture conditions and media preparation: MS medium (Murashige & Skoog, 1962) was thoroughly used in tissue culture experiments. The pH was adjusted to 5.7-5.8 using NaOH and HCl if needed. Medium was then solidified with 0.8% agar (8g/l) and autoclaved at 121°C for 20 minutes. It was poured into test tubes ($1/4^{th}$) and petri dishes in laminar flow (Hartman *et al.*, 1994). Cultures were then shifted to growth chamber at 25°C with 16 hours photoperiod supplemented with 2000-2500 lux light intensity for a week. Best media escaped from any contamination was selected for inoculation.

Direct regeneration: MS medium was also fortified with twelve different concentrations of three hormonal combinations (MS + BAP, MS+BAP+NAA and MS+ BAP+ Kin) for possible achievement of best medium for directly obtaining shoots from stem node, leaf and shoot tip explants.

Rooting of regenerated plantlets: Healthy and profusely regenerated plantlets either from direct or indirect organogenesis were separated and cultured on ½ strength MS medium fortified with various potentially suspected concentrations of root inducing hormones IBA and NAA (0, 0.5 1, 1.5 mg/l).

Acclimatization: Well rooted plantlets were finally subjected to hardening or acclimatization in the best potting media i.e., Sand + Soil + Farmyard (1:1:1) amongst Soil + Sand (1:1), Sand + Peat (2:1), Red soil + Sand (1:1), Sand + Soil + Rice bran ash (1:1:1) and Sand + Soil + Farmyard (1:1:1) after washing the roots with water to remove media debris. Pots were placed in green house covered with nylon bags to provide humidity. Bags were gently perforated once a day until no condensation observed (Baskaran & Jayabalan, 2005). After removing the bags they were shifted to field.

Statistical analysis: All the experiments were arranged up in a randomized complete block design. Values were represented as mean ± SE of three randomly selected readings of 10 replicates per treatment in three repeated experiments. The data was subjected to analysis of variance (ANOVA) to detect significant difference between means. Means differing significantly were compared using LSD at P = 0.05. All the statistical analysis was done by using STATISTICS 8 (version 8.1) analytical software USA and Microsoft excel 2007 software.

Results and Discussion

Direct regeneration: Direct regeneration can lead to potential and more often meristematic plant parts to morphogenized into buds, shoots, roots, leaf or even to flower. This technique actually ensures very less clonal variations in plants as compared to indirect organogenesis which mostly ends up in more or less clonal variations (Cavusoglu *et al.*, 2011).

Effect of BAP on shoot induction % via direct organogenesis: The MS medium with various concentrations of BAP was tested for possible shoot induction (Table 1). Response of explants according to increased concentration of BAP was somewhat un-symmetric or irregular. Both of the two concentrations *i.e.* D2 and D3 were found to be significantly considerable but for leaf and for stem node and shoot tip. D2 showed 71% induction for leaf and D3 showed 80 and 81% for others, respectively. Similar results were obtained by Mukhtar *et al.* (2005) and Sivanandhan *et al.* (2011) describing the same concentrations as useful for direct shoot bud regeneration.

Effect of BAP + NAA on shoot induction % via direct organogenesis: Two hormones viz., BAP and NAA were synergistically used in different treatments named $D_{5=}$ MS (1.0 + 0.1 mg/l), $D_{6=}$ MS (1.3 + 0.1 mg/l), $D_{7=}$ MS (1.6 + 0.1 mg/l), $D_{8=}$ MS (1.9 + 0.1 mg/l) to find the maximum shoot induction % in all three explants. The data is arranged in Table 1 with statistic evaluation of the differences between means of treatments as well as of explants type at $p< 0.05$. From the Table 1, it is obvious that D_8 MS (1.9 + 0.1 mg/l) was most successful combination amongst the four used. About 91% of the test tubes having stem node as well as of leaf explants showed excellent response with no significant difference (Figs. 1 & 2). While shoot tip showed somewhat moderate (81%). Except this concentration all were not significant regarding treatment and explants and proved to be less responsive to increase in the concentration. Lowest results were obtained at very less concentration of MS fortified with BAP (1.0mg/l) and NAA.

Our results are in very much agreement with only known micropropagation protocol of *S. villosum* (Hussein & Aklan, 2011) regarding the concentration of BAP and NAA and type of the explants used. They also observed non significant increase in shoot induction improvement with increasing concentration up to the best one. Bhatti *et al.* (2014) also affirmed our observations in their study on *Solanum melongena*. Our results are in disagreement with Pandhure *et al.* (2010) as they reported BAP and NAA as best combination for said purpose in *Solanum nigrum*. Our results also showed more induction (91%) in both leaf and stem node compared to 90% in previous findings. Previously only leaf was reported as best explants although both were used. We obtained maximum proliferation throughout in 16 hours photoperiod not in dark. In this regard our results are deviating from (Hussein & Aklan, 2011) showing more precision of handling experiment at every stage with some modifications in methodology adopted by us and also make our work distinguished, elaborative and fruitful addition of the previously known protocol.

Table 1. Effect of different medium composition with various concentrations on shoot induction percentage *via* direct organogenesis in *Solanum villosum* (Culture's age = 5 weeks).

Treatment	Media	Conc. (mg/l)	No. of explants	No. of explants showing shoot induction		
				Stem node	Leaf	Shoot tip
D_1	MS + BAP	0.5	10	5.0 ± 0.04^{g}	5.1 ± 0.04^{g}	4.3 ± 0.25^{h}
D_2		1.0	10	7.9 ± 0.04^{b}	7.1 ± 0.04^{c}	5.7 ± 0.04^{e}
D_3		1.5	10	8.0 ± 0.08^{ab}	6.7 ± 0.15^{d}	8.1 ± 0.07^{a}
D_4		2.0	10	5.4 ± 0.20^{f}	4.3 ± 0.04^{h}	5.0 ± 0.04^{g}
D_5	MS+BAP+NAA	1.0 +0.1	10	6.6 ± 0.08^{g}	6.7 ± 0.04^{g}	6.6 ± 0.08^{g}
D_6		1.3 +0.1	10	7.0 ± 0.04^{ef}	6.9 ± 0.07^{f}	7.3 ± 0.11^{d}
D_7		1.6 +0.1	10	7.2 ± 0.04^{de}	7.1 ± 0.04^{e}	7.9 ± 0.04^{c}
D_8		1.9 +0.1	10	9.1 ± 0.12^{a}	9.1 ± 0.12^{a}	8.1 ± 0.07^{b}
D_9	MS+ BAP+ Kin	0.5 +0.2	10	6.1 ± 0.04^{d}	6.5 ± 0.20^{cd}	6.6 ± 0.18^{cd}
D_{10}		0.5 +0.3	10	6.4 ± 0.10^{cd}	6.4 ± 0.27^{d}	6.6 ± 0.24^{cd}
D_{11}		0.5 +0.4	10	6.7 ± 0.02^{cd}	6.5 ± 0.32^{cd}	6.7 ± 0.16^{cd}
D_{12}		0.5 +0.5	10	7.9 ± 0.39^{a}	7.4 ± 0.45^{ab}	7.0 ± 0.04^{bc}

Legend: D_1 to D_{12} represents (conc. mg/l). Values represent mean ± SE of three randomly selected readings of 10 replicates per treatment in three repeated experiments. Means followed by same letters are not significantly different by the LSD test at the 5% level ($p \leq 0.05$)

Table 2. Effect of different mediums with their concentrations on shoot number (per explants) by means of direct organogenesis in *Solanum villosum* (Culture's age = 5 weeks).

Treatment	Media	Conc. (mg/l)	No. of explants	No. of shoots per explants		
				Stem node	Leaf	Shoot tip
D_1	MS + BAP	0.5	10	1.9 ± 0.07^{c}	1.7 ± 0.41^{c}	2.0 ± 0.70^{c}
D_2		1.0	10	4.7 ± 0.41^{b}	1.5 ± 0.02^{c}	2.3 ± 1.08^{c}
D_3		1.5	10	7.6 ± 0.39^{a}	7.3 ± 0.41^{a}	7.0 ± 0.61^{a}
D_4		2.0	10	1.7 ± 0.41^{c}	1.5 ± 0.61^{c}	2.0 ± 0.70^{c}
D_5	MS+BAP+NAA	1.0 + 0.1	10	2.0 ± 0.70^{cd}	2.0 ± 0.70^{cd}	1.0 ± 0.70^{d}
D_6		1.3 + 0.1	10	3.3 ± 1.47^{bc}	4.2 ± 0.20^{b}	2.0 ± 0.70^{cd}
D_7		1.6 + 0.1	10	4.2 ± 0.20^{b}	7.6 ± 0.39^{a}	1.3 ± 1.08^{d}
D_8		1.9 + 0.1	10	7.6 ± 0.39^{a}	9.1 ± 0.12^{a}	2.3 ± 1.08^{cd}
D_9	MS+ BAP+ Kin	0.5 + 0.2	10	2.3 ± 1.08^{ab}	1.5 ± 0.35^{b}	2.3 ± 1.78^{ab}
D_{10}		0.5 + 0.3	10	1.7 ± 0.41^{b}	2.7 ± 0.82^{ab}	3.3 ± 1.47^{ab}
D_{11}		0.5 + 0.4	10	2.0 ± 0.70^{ab}	2.7 ± 0.82^{ab}	3.7 ± 1.08^{ab}
D_{12}		0.5 + 0.5	10	4.2 ± 0.20^{a}	3.3 ± 0.82^{ab}	3.3 ± 0.82^{ab}

Legend: D_1 to D_{12} represents (conc. mg/l). Values represent mean ± SE of three randomly selected readings of 10 replicates per treatment in three repeated experiments. Means followed by same letters are not significantly different by the LSD test at the 5% level ($p \leq 0.05$)

Fig. 1. Shoot bud organogenesis from leaf.

Fig. 3. Shoot proliferation.

Fig. 2. Organogenesis from stem node.

Fig. 4. Rooting from excised shoots.

Effect of BAP+Kin on shoot induction %via direct organogenesis: By keeping the concentration of BAP constant (0.5mg/l) four different concentrations of Kin (0.2, 0.3, 0.4, 0.5mg/l) were employed in MS medium for shoot induction in all explants aforementioned (Table 1). It was noticed that only one concentration D_{12} containing equal concentration of BAP and Kin was able to give somewhat appreciable but not much desired results. In *W. coagulans*, Jain *et al.* (2009) reported the direct regeneration by means of shoot tip and nodal segments as explants cultured on MS+ BA (0.5 mg L-1), Kin (0.5 mg L-1). Our results are quite opposite to some findings (Silva *et al.*, 2005; Germana *et al.*, 2011).

Effect of different medium composition on shoot number per explants via direct organogenesis: After 5 weeks taken by explants for direct shoot bud induction followed by emergence of little shoots. Cultures were shifted to fresh multiplication or proliferation MS media with same combinations. No. of shoots were carefully counted per explants for all the three explants in all three organogenic MS medium nourished with BAP, BAP+NAA and BAP+ Kin (Table 2). It was noticed that explants showed significant number of shoots (Fig. 3) in D3 with 7.6, 7.3 and 7 mean number of shoots. Mukhtar *et al*., 2005 and Sivanandhan *et al.* (2011) also confirmed our findings. It was observed that out of 4 treatments of MS media supplemented with BAP (1.0, 1.3, 1.6, 1.9 mg/l) and NAA(0.1 MG/L) only D_8 was able to proliferate well in stem node and leaf explants with 7.6 and 9.1 mean number of shoots with no significant differences at $p< 0.05$. These findings are in agreement with Hussein & Aklan (2011), who proposed the same trend. Further increase in concentration could not significantly induce shoot multiplication. Only D_{12} showed somewhat considerable results (4.2, 3.3 and 3.3). Results are comparable with Jain *et al.* (2009).

Table 3. Effect of NAA and IBA with their concentrations on root initiation percentage in *Solanum villosum* (Culture's age = 9 weeks).

Treatment	Media	Conc. (mg/l)	No. of explants	No. of test tubes showing rooting		
				Stem node	Leaf	Shoot tip
Rt_1	MS +IBA	0.0	10	6.7±0.40[bc]	7.0±0.07[ab]	7.8±0.15[a]
Rt_2		0.5	10	4.6±0.25[f]	5.1±0.16[def]	5.7±0.41[cdef]
Rt_3		1.0	10	4.9±0.16[ef]	5.3±0.86[def]	5.8±0.35[cde]
Rt_4		1.5	10	5.7±0.30[cde]	5.4±0.78[def]	6.2±0.74[bcd]
Rt_5	MS+NAA	0.0	10	6.7±0.40[b]	7.0±0.07[b]	7.8±0.15[a]
Rt_6		0.5	10	5.1±0.18[de]	4.9±0.04[e]	5.1±0.07[de]
Rt_7		1.0	10	5.3±0.11[de]	5.1±0.15[de]	5.4±0.07[cd]
Rt_8		1.5	10	5.4±0.07[cd]	5.2±0.07[de]	5.6±0.04[c]

Legend: Rt_1 to Rt_8 represents (conc. mg/l). Values represent mean ± SE of three randomly selected readings of 10 replicates per treatment in three repeated experiments. Means followed by same letters are not significantly different by the LSD test at the 5% level ($p \leq 0.05$)

Table 4. Effect of various potting mixes on the survival of micro plants during hardening of *Solanum villosum*.

Tr. No.	Potting media	Survival % of micro plants	Age of Micro plant (Week)
1.	Soil + Sand (1:1)	70	10
2.	Sand + Peat (2:1)	40	10
3.	Red soil + Sand (1:1)	50	10
4.	Sand + Soil + Rice bran ash (1:1:1)	60	10
5.	Sand + Soil + Farmyard (1:1:1)	100	10

Legend: Values shown were proportions of three replicates of 30 micro plants

Rooting: Roots were not initiated during shoot multiplication in the shoot induction media. So, individual shoots were aseptically transferred to half strength MS with no hormones. The MS medium was supplemented with IBA and NAA in four concentrations each to find out best medium for root induction. Results (Table 3, Fig. 4) showed that MS hormonal medium was more successful in inducing roots as compared to medium supplemented with auxin. Our results are in strong agreement with Hussein & Aklan, (2011), who also reported same finding in an attempt to induce rooting in *S. villosum.* Other members of its family also showed similar behavior such as in *S. donianum* (Connor-Sánchez *et al*., 2010) and *Withania sominifera* (Nathiya *et al*., 2013). This medium induced 67, 70 and 78% rooting in shoots regenerated via node, leaf and shoot tip explants, respectively. Addition of auxins also had effect on rooting but was slightly less significant as compared to their presence free media. Same kind of setup was used by Bhatti *et al*. (2014) with same observations in *S. melongena*. It was observed that although NAA was more effective rooting medium as compared to IBA for *S. villosum,* but increase in the concentrations of both auxins resulted in increased frequency of rooting in all types of explants regenerated shoots. Our observations are in line of Caraballo *et al.* (2010) and Savita *et al.* (2010).

Acclimatization/hardening: Table 4 is evident of our observation that Sand + Soil + Farmyard (1:1:1) was the best potting medium, in which all plantlets were successfully survived (100%). Rest of the media showed less survival percentages (Table 4). Our findings regarding the best acclimatization media for *S. villosum* are in strong conformity with many other studies (Baskaran & Jayabalan, 2005; Ghauri *et al*., 2013). Some close relatives of this species showed best survival in other types of media which is contradicted to our results (Loc & Kiet, 2011; Arockiasamy *et al*., 2002).

Conclusion

Solanum villosum was successfully propagated through leaf and stem node by using successful combination (1.9 BAP + 0.1 NAA mg/l). This study has opened gateways for producing cell, tissue and suspension cultures of *S. villosum.* Scientific efforts are further needed to prove medicinal potential of this plant in order to further attract tissue culturists and botanists to propagate it at a large scale considering its importance.

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