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PLANT REGENERATION THROUGH CALLUS INDUCTION ON MEDICINAL HERB Viola *odorata* - ROLE OF PLANT GROWTH REGULATORS AND EXPLANTS

SUMMARY

Viola odorata is a hardy herbaceous flowering perennial medicinal plant mainly used as an herbal cure in the diabetes and cancer, which have a low germination rate under normal laboratory conditions because of hard seed coat and thermal dormancy. To examine the effects of some plant growth regulators on callus induction and plant regeneration of *V. odorata*, various explants such as leaf, petiole and root pieces were transferred to Petri dishes containing MS medium supplemented by different concentration and combination of plant growth regulators NAA, 2,4-D and KIN. The highest frequency of callus induction (80%) was occurred in MS media containing NAA (2 mg l^{-1}) + 2, 4-D (2.5 mg l^{-1}) on leaf explants. The best treatment for rooting was the medium containing NAA (3 mg l^{-1}) + 2, 4-D (2.5 mg l^{-1}). After transfer the calli to regeneration media, results showed the best response to regeneration was occurred in MS media containing GA3 (0.5 mg l^{-1}) + TDZ (2 mg l^{-1}) also GA3 (1 mg l^{-1}) + TDZ (2 mg l^{-1}). Maximum mean number of shoots and highest length of shoots were obtained in MS media supplemented with GA3 (0.5 mg. l^{-1}) + TDZ (2 mg. l^{-1}).

Keywords: Callus; Petioles; Shoot regeneration; TDZ; *Viola odorata*

INTRODUCTION

There is a growing concern worldwide about over exploitation and reducing the earth's natural resources, especially plant biodiversity. So, the need for conservation is high and of main importance to preserve the plant diversity heritage, especially the rare and endemic medicinal plants for posterity (Cragg, 2001). *Viola odorata* known as sweet violet, is considered as rare and endemic perennial plant to Europe and Asia and belongs to the genus viola (*MALPIGHIALES*: Violaceae) which, includes more than 400 species (Mabberley, 1987). Sweet violet is one of the most important medicinal plant

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Abbreviations: GA3; KIN, Kinetin; MS, Murashige and Skoog medium (1962); NAA, Naphthalene acetic acid; PGRs, Plant Growth Regulators; TDZ, Thidiazuron.

Note: The authors declare that they have no conflicts of interest. Authorship Form signed online.

used folklore therapy for curing various ailments like bronchitis, common digestive disorders, post-operative tumor metastasis, diabetes and cancer. Phytochemically, different groups of compounds have been isolated from various species of this genus like cyclotides, flavonoids, alkaloids and triterpenoids (Darwin, 2010). Some of them already have been scientifically accepted as antifungal, antibacterial, anticancer, antioxidant, antiasthmatic, anti-inflammatory, anti-HIV and antipyretic agents (Ireland et al., 2006; Ebrahimzadeh et al., 2010; Gustafson, 2004). Recently, a new herbal drug "ODORATA" which formulated into syrup is synthesized based on active constituents of *V. odorata*. This has increased the importance of developing tissue culture methods to make easy large-scale production of true-to-type plants and for improving the species using genetic engineering techniques. It has been shown that callus culture can be used as an effective method for multiplication of medicinal plants (Castillo and Jordan, 1997). The germination rate of this medicinal plant is low because of sever seed dormancy (lord, 1983). The industrial interest in needs the development of sweet violet *in vitro* method to speed up the propagation rate. There is the little information about plant regeneration of this species (Tobyn, 2011), but callus induction from petiole explants and regeneration of multiple shoots in *Viola serpens* have been reported (Vishwakarma et al., 2013). So, the present work has been undertaken to evaluate the effects of plant growth regulators (PGRs) on callus induction from leaf, petiole and root and *in vitro* propagating *V. odorata*.

MATERIAL AND METHODS

Plant material. This study was conducted in the Plant Tissue Culture Department of Agricultural Biotechnology Research Institute of Iran in 2013. Seeds were collected from the Medicinal Plant Collection of Agriculture Research Center, Isfahan, Iran. Seeds were surface sterilized, pretreated and sown on ¼ MS medium according to our previous report Barekat et al., (2013).

Callus establishment and condition. Three kinds of explants including root (1 cm), petiole (1 cm) and leaf (0.5 × 0.5 cm^2) were taken from *in vitro* seedlings of germinated seeds. They were slightly wounded and four of them horizontally placed on Petri dishes containing solidified-MS medium fortified with various concentration of 2,4-D (0, 0.5, 1, 1.5 and 2 mg l^{-1}) alone or in combination with NAA (0, 0.5, 1, 1.5 and 2 mg.l^{-1}) and KIN (0, 0.5 and 1 mg.l^{-1}). Each Petri dishes were replicates 4 times and incubated at 25 ± 1°C in total darkness for 3 weeks and then, transferred to 16/8 hours (light: dark) photoperiod at ~3000 lux and 25 ± 1°C. After another 3 weeks, callus induction frequency (%) = number of explants producing calli/number of explants plated x 100 and shoot or root induction frequency (%) were recorded. All experiments were done following a factorial experiment based on completely randomized design with equal replications.

Shoot multiplication, rooting and elongation. For shoot multiplication and further root induction, 4 callus formed on the used explants were isolated and

transferred onto culture bottles containing solid MS regeneration medium supplemented by different levels of TDZ (0, 1, 2, 3 and 4 mg.l^{-1}) combined with GA3 at (0, 0.5, 1, 1.5 and 2 mg.l^{-1}) and IBA (1 mg.l^{-1}) for 4 weeks at 16 hours photoperiod at ~3000 lux and 25˚ ± 10˚C. The regeneration frequency (%) = number of plants regenerated/number of calli plated x 100, and mean number and length of micro-shoots frequency (%) were measured. Each culture bottle (treatment) was replicated 4 times. 2, 4-D (2.5 mg.l^{-1}) + NAA (2 mg.l^{-1}) on leaf explants; further growth and development of green callus after 3 weeks on transferring to photoperiod (light: dark) 16/8 hours.

Hardening. After two months of incubation in regeneration medium, the well rooted plants were transferred onto plastic pots filled with peat + perlite (2:1). Finally hardened seedlings were further transferred into plastic bucket containing fertilized garden soil and grown in greenhouse at 22±2°C and 50-60% relative humidity and irrigated regularly.

Data analysis. Data were analyzed using the SAS version 9.1. When the ANOVA indicated significant treatment effects (5 or 1%) based on the F-test, Least Significant Difference test (P<0.5) was used to find out which treatments were significantly different from other treatments.

RESULTS AND DISCUSSION

Callus induction, regeneration and rooting. Callusing wasn't occurred in all treatments and those which produced any results, were not shown in Table 1. Callus induction frequency in response to different combinations of plant growth regulators and explants types is summarized in Table 1.

Table 1.Interaction effects of different Explants and PGRs on % callus induction, regeneration and rooting of *V. odorata*

Plant Growth Regulators (mg.l^{-1})	% Callus induction		% Regeneration		% Rooting	
	Petiole	*Leaf*	*Petiole*	*Leaf*	*Petiole*	*Leaf*
2,4-D(0) + NAA (1)	5.00 ^{hi}	10.00 $^{g-i}$	5.00 ^{ij}	10.00 $^{h-j}$	0.00 ^{f}	*Leaf*
2,4-D(0) + NAA (1.5)	5.00 ^{hi}	10.00 $^{g-i}$	5.00 ^{ij}	10.00 $^{h-j}$	5.00 ^{ef}	5.00 ^{ef}
2,4-D(0) + NAA (2)	5.00 ^{hi}	10.00 $^{g-i}$	5.00 ^{ij}	5.00 ^{ij}	0.00 ^{f}	5.00 ^{ef}
2,4-D(0) + NAA (2.5)	5.00^{hi}	5.00 ^{hi}	0.00 ^{j}	5.00 ^{ji}	0.00 ^{f}	5.00 ^{ef}
2,4-D(0) + NAA (3)	5.00 ^{hi}	5.00 ^{hi}	0.00 ^{j}	5.00 ^{ji}	0.00 ^{f}	5.00 ^{ef}
2,4-D(0.5) + NAA (0)	10.00 $^{g-i}$	15.00 $^{f-i}$	10.00 $^{h-j}$	10.00 $^{h-j}$	0.00 ^{f}	0.00 ^{f}
2,4-D(0.5) + NAA (0.5)	10.00 $^{g-i}$	20.00 $^{e-h}$	10.00 $^{h-j}$	15.00 $^{g-j}$	5.00 ^{ef}	0.00 ^{f}
2,4-D(0.5) + NAA (1.5)	25.00 $^{d-g}$	40.00 ^{cd}	15.00 $^{g-j}$	30.00 $^{d-g}$	10.00 ^{de}	5.00 ^{ef}
2,4-D(0.5) + NAA (2)	5.00 ^{hi}	10.00 $^{g-i}$	5.00 ^{ij}	5.00 ^{ij}	0.00 ^{f}	10.00 ^{de}
2,4-D(0.5) + NAA (2.5)	15.00 $^{f-i}$	20.00 $^{e-h}$	10.00 $^{h-j}$	15.00^{g-j}	5.00 ^{ef}	5.00 ^{ef}
2,4-D(0.5) + NAA (3)	10.00 $^{g-i}$	15.00 $^{f-i}$	10.00 ^{hij}	10.00 $^{h-j}$	5.00 ^{ef}	5.000 ^{ef}
2,4-D(1) + NAA (0)	5.00 ^{hi}	5.00 ^{hi}	5.00 ^{ij}	5.000 ^{ij}	0.00 ^{f}	5.00 ^{ef}
2,4-D(1) + NAA (0.5)	5.00 ^{hi}	10.0 $^{g-i}$	5.00 ^{ij}	10.00 $^{h-j}$	0.00 ^{f}	0.00 ^{f}
2,4-D(1) + NAA (2)	35.00 $^{c-e}$	45.00 ^{bc}	5.00 ^{ij}	15.00 $^{g-j}$	10.00 ^{ed}	0.00 ^{f}
2,4-D(1) + NAA (2)	5.00^{hi}	35.00 $^{c-e}$	5.00^{ij}	20.00 $^{f-i}$	0.00 ^{f}	15.00 ^{cd}
2,4-D(1.5) + NAA (0)	5.000^{hi}	5.00^{hi}	5.00 ^{ij}	5.000 ^{ij}	5.00 ^{ef}	10.00 ^{ed}
2,4-D(1.5) + NAA (1)	20.00 $^{e-h}$	10.00 $^{g-i}$	10.00 $^{h-j}$	5.000 ^{ij}	5.00 ^{ef}	0.00 ^{f}
2,4-D(1.5) + NAA (1.5)	40.00 ^{cd}	30.00 $^{c-f}$	10.00 ^{hij}	15.00 $^{g-j}$	15.00 ^{cd}	0.00 ^{f}
2,4-D(1.5) + NAA (3)	5.00 ^{hi}	60.00 ^{b}	5.00 ^{ij}	20.00 $^{f-i}$	0.00 ^{f}	10.00 ^{de}
2,4-D(2) + NAA (1.5)	5.00 ^{hi}	10.00 $^{g-i}$	0.00 ^{j}	10.00 $^{h-j}$	0.00 ^{f}	5.00 ^{ef}

2,4-D(2) + NAA (2)	10.00 $^{g-i}$	5.00 ^{hi}	5.00 ^{ij}	5.00 ^{ij}	5.00 ^{ef}	5.00 ^{ef}
2,4-D(2) + NAA (2.5)	5.00 ^{hi}	10.00 $^{g-i}$	5.00 ^{ij}	10.00 $^{h-j}$	0.00 ^{f}	5.00 ^{ef}
2,4-D(2) + NAA (3)	10.00 $^{g-i}$	5.00 ^{hi}	10.00 $^{h-j}$	5.00 ^{ij}	0.00 ^{f}	5.00 ^{ef}
2,4-D(2.5) + NAA (0)	10.0 $^{g-i}$	15.00 $^{f-i}$	10.00 $^{h-j}$	15.00 $^{g-j}$	0.00 ^{f}	5.00 ^{ef}
2,4-D(2.5) + NAA (0.5)	40.00 ^{cd}	15.00 $^{f-i}$	15.00 $^{g-j}$	15.00 $^{g-j}$	10.00 ^{de}	0.00 ^{f}
2,4-D(2.5) + NAA (1)	5.00 ^{hi}	60.00 ^{b}	5.00 ^{ij}	5.00 ^{ij}	5.00 ^{ef}	5.00 ^{ef}
2,4-D(2.5) + NAA (1.5)	60.00 ^{b}	10.00 $^{g-i}$	5.00 ^{ij}	10.00 $^{h-j}$	40.00 ^{b}	40.00 ^{b}
2,4-D(2.5) + NAA (2)	30.00 $^{c-f}$	80.00 ^{a}	20.00 $^{f-i}$	10.00 $^{h-j}$	25.00 ^{bc}	25.00 ^{bc}
2,4-D(2.5) + NAA (2.5)	45.00 ^{cb}	40.00 ^{cd}	0.00 ^{j}	20.00 $^{f-i}$	40.00 ^{b}	25.00 ^{bc}
2,4-D(2.5) + NAA (3)	5.00 ^{hi}	60.00 ^{b}	0.00 ^{j}	20.00 $^{f-i}$	0.00 ^{f}	55.00 ^{a}
2,4-D(3) + NAA (0)	5.00 ^{hi}	5.00 ^{hi}	0.00 ^{j}	5.00 ^{ij}	0.00 ^{f}	5.00 ^{ef}
2,4-D(3) + NAA (0.5)	10.00 $^{g-i}$	5.00 ^{hi}	10.00 $^{h-j}$	5.00 ^{ij}	0.00 ^{f}	0.00 ^{f}
2,4-D(3) + NAA (1)	5.00 ^{hi}	20.00 $^{e-h}$	5.000^{ij}	15.00 $^{g-j}$	0.00 ^{f}	0.00 ^{f}
2,4-D(3) + NAA (1.5)	25.00 $^{d-g}$	10.00 $^{g-i}$	20.00 $^{f-i}$	10.00 $^{h-j}$	0.00 ^{f}	0.00 ^{f}
2,4-D(3) + NAA (2)	10.00 $^{g-i}$	30.00 $^{c-f}$	10.00 $^{h-j}$	15.00 $^{g-j}$	0.00 ^{f}	0.00 ^{f}
2,4-D(3) + NAA (2.5)	15.00 $^{f-i}$	10.00 $^{g-i}$	10.00 $^{h-j}$	20.00 $^{f-i}$	0.00 ^{f}	0.00 ^{f}
2,4-D(3) + NAA (3)	30.00 $^{c-f}$	20.0 $^{e-h}$	20.00 $^{f-i}$	15.00 $^{g-j}$	0.00 ^{f}	0.00 ^{f}
2,4-D(2.5) + NAA (0) + KIN (0.5)	10.00 $^{g-i}$	45.00 ^{bc}	20.00 $^{f-i}$	40.00 $^{c-e}$	0.00 ^{f}	0.00 ^{f}
2,4-D(2.5) + NAA (0.5) + KIN (0.5)	20.00 $^{e-h}$	30.00 $^{c-f}$	20.00 $^{f-i}$	45.0 $^{b-d}$	5.00 ^{ef}	0.00 ^{f}
2,4-D(2.5) + NAA (1) + KIN (0.5)	30.00 $^{c-f}$	20.00 $^{e-h}$	40.00 $^{c-e}$	35.00 $^{c-f}$	10.00 ^{de}	0.00 ^{f}
2,4-D(2.5) + NAA (2) + KIN (0.5)	30.00 $^{c-f}$	45.00 ^{cb}	25.00 $^{e-h}$	45.00 ^{cd}	5.000 ^{ef}	5.00 ^{ef}
2,4-D(2.5) + NAA (2.5) +KIN (0.5)	10.00 $^{g-i}$	45.00 ^{cb}	10.00 $^{h-j}$	35.00 $^{c-f}$	0.00 ^{f}	25.00 ^{bc}
2,4-D(2.5) + NAA (0) + KIN (1)	20.00 $^{e-h}$	45.00 ^{cb}	50.00 ^{bc}	67.00 ^{a}	0.00 ^{f}	15.00 ^{cd}
2,4-D(2.5) + NAA (0.5) + KIN (1)	15.00 $^{f-i}$	30.00 $^{c-f}$	25.00 $^{e-h}$	40.00 $^{c-e}$	5.00 ^{ef}	0.00 ^{f}
2,4-D(2.5) + NAA (1) + KIN (1)	20.00^{e-h}	20.00 $^{e-h}$	40.00 $^{c-e}$	50.00 ^{b}	10.00 ^{de}	0.00 ^{f}
2,4-D(2.5) + NAA (2) + KIN (1)	10.00 $^{g-i}$	30.00^{c-f}	30.00 $^{d-g}$	40.00 $^{c-e}$	5.00 ^{ef}	10.00 ^{de}
2,4-D(2.5) + NAA (3) + KIN (1)	30.00 $^{c-f}$	20.00 $^{c-f}$	30.00 $^{d-g}$	30.00 $^{d-g}$	0.00 ^{f}	10.00 ^{de}
2,4-D(2)	10.00 $^{g-i}$	10.00 $^{g-i}$	0.00 ^{j}	0.00 ^{j}	0.00 ^{f}	5.00 ^{ef}
2,4-D(3)	5.00 ^{hi}	10.00 $^{g-i}$	0.00 ^{j}	0.00 ^{j}	0.00 ^{f}	0.00 ^{f}
2,4-D(4)	5.00 ^{hi}	10.00 $^{g-i}$	0.00 ^{j}	0.00 ^{j}	0.00 ^{f}	0.00 ^{f}

Means followed by different letters are significantly different ***at p = 0.05*** *according to LSD test.*

Results showed the callus induction frequency varied significantly ($p<0.05$) depending on plant growth regulators and kinds of explants. The highest frequency of callus induction (80%) was observed in MS media containing 2,4-D (2.5 mg l^{-1}) + NAA (2 mg l^{-1}) on leaf explants (Figure 1, a-d). Also on the media containing 2,4-D (2.5 mg.l^{-1}) + NAA (1mg.l^{-1}), 2,4-D (2.5mg.l^{-1}) + NAA (1.5 mg.l^{-1}) and 2,4-D (2.5mg.l^{-1}) + NAA (3mg.l^{-1}) the frequency of callus induction was high, but the difference among these treatments was not significant. NAA and 2, 4-D are commonly used for callus induction in various systems (Davidovic et al 2015).As mentioned above, 2, 4-D in combination with NAA caused the maximum callus induction in explants. These findings are in support of the results obtained by Wang and Bao (2007). Similarly, in *Astragalus polemoniacus*, leaf and petiole explants produced calli on media containing NAA and BA (Mirici, 2004). Naeem et al., (2013) also reported that 2.5 mg.l^{-1} BA and 0.15 mg.l^{-1}2, 4-D could induce high frequency of calli from *Viola odorata* leaves, stem and petioles explants. Anzidei et al., (2000) reached callus by *in vitro* culturing leaf segments of *Foeniculum vulgare* using 2, 4-D.The callus which induced in dark condition was yellow and friable but, those in light condition were green and friable (Figure 1, a and c). It was found that light condition which promotes higher rate of chlorophylls synthesize could be the possible reason (Zhong, 1991). Some of induced calli were reasonably rooted on callus induction media.

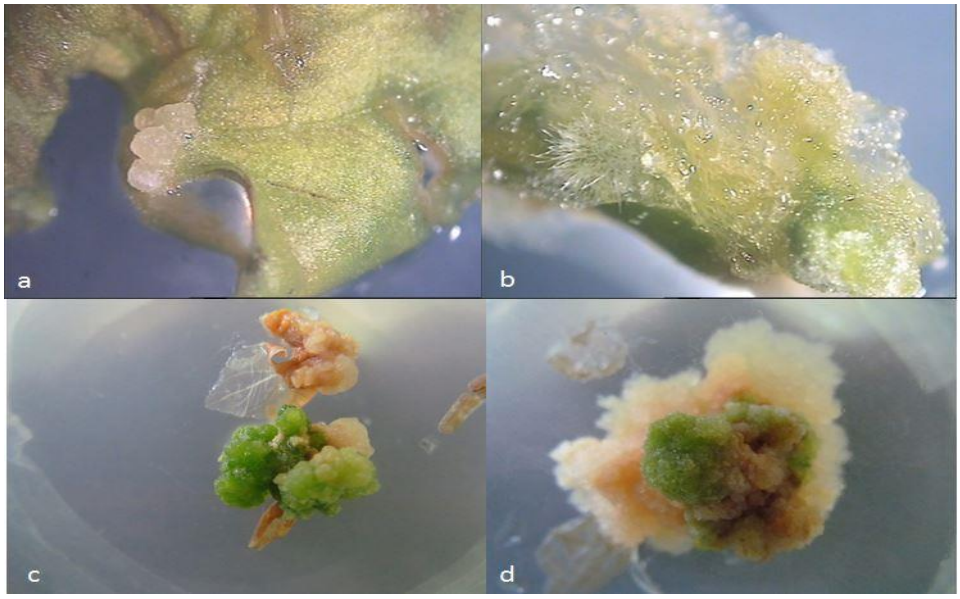


Figure 1. Initiation of callus tissues (yellow- friable) incubated at dark (a and b) after 21-30 days in MS media containing2,4-D (2.5 mg.l^{-1}) + NAA (2 mg.l^{-1}); further growth and development of callus (green-friable) after another 3 weeks on leaf explants (c and d).

The highest percentage (55%) of rooting was observed on MS media supplemented with 2, 4-D (2.5mg.l^{-1}) + NAA (3mg.l^{-1}). Previously Jafarkhani Kermani et al., (2010) achieved rooting by *in vitro* culturing leaves of *Rosa persica* using NAA in combination with IBA. The promoting effect of auxin and cytokinin combinations on organogenic differentiation has been well documented in several systems (Dimech, 2007). Similarly the effects of 2, 4-D combined with NAA or KIN for successful shoot development from callus observed in our study. The best response for shoot regeneration (67%; Figure 2, a-d) was observed on callus MS-based media containing (1 mg.l^{-1}) KIN + (2.5 mg.l^{-1}) 2, 4-D followed by KIN (1 mg.l^{-1}) + 2, 4-D (2.5 mg.l^{-1}) +NAA (1 mg.l^{-1}). These results were accordance with some previous studies which show the using of combination of 2, 4-D and NAA caused the highest rate of callus induction and regeneration (Nikam, 1999).

The effects of explants types on percentages of callus induction, regeneration and rooting. Investigation of the effect of explants on the callus induction, regeneration and rooting of plantlets showed the applying of leaf explants was better than the other types of explants and by use of leaf segments as explants, percentage of callus induction, regeneration and rooting of plantlets were16.34, 13.95 and 4.05 respectively (Figure3). These results agree to available reports for *Suffruitcosum indiuom* (Arunkumar et al., 2011). Also the favorable effect of using leaf explants for callus induction has already been reported by Stephan and Jayabalan (2000).Similarly, callus induction potential was found higher in leaves compared to stem and petioles of *V. odorata* in which callus was found throughout the leaf disk while in stem and petiole, callus was localized to the tips and terminal parts (Wijowska, 1999).

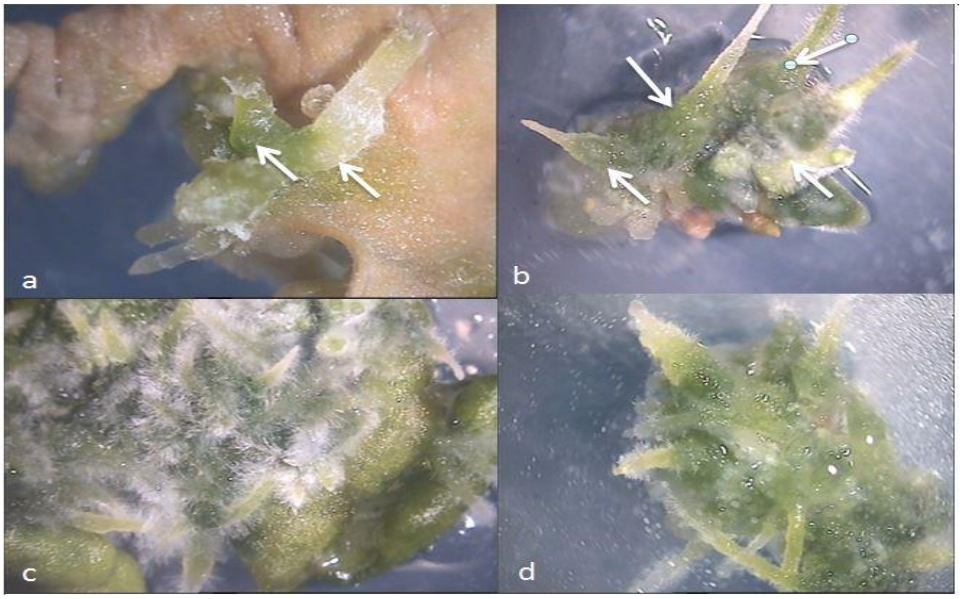
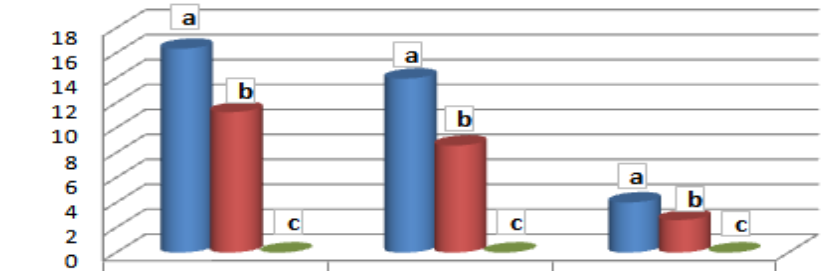


Figure 2. Shoot bud initiation on 6 weeks old calli derived from leaf explants (a-d) on same callus media containing NAA (2 mg.l^{-1}) + 2,4-D (2.5 mg.l^{-1}).



	% callus induction	% regeneration	% rooting
leaf	16.34	13.95	4.02
petiol	11.26	8.58	2.61
root	0	0	0

Figure 3.The effects of explants types on percentages of callus induction, regeneration and rooting of *V.odorata*.

Regeneration, shoot multiplication and elongation. After successful callus induction, the calli derived from all explants were sub-cultured on regeneration media. As shown in Figure 4 (a-b), the regeneration frequency varied among different treatments and the best response for regeneration was observed in MS media containing GA3 (0.5 mg.l^{-1}) + TDZ (2 mg.l^{-1}) and GA3 (1mg.l^{-1}) + TDZ (2mg.l^{-1}).The effect of various plant growth regulators on mean number and length of callus derived micro-shoots was also researched. According to Table 2, the number and length of shoots varied significantly depending on combination of TDZ and GA3 ($p<0.05$).

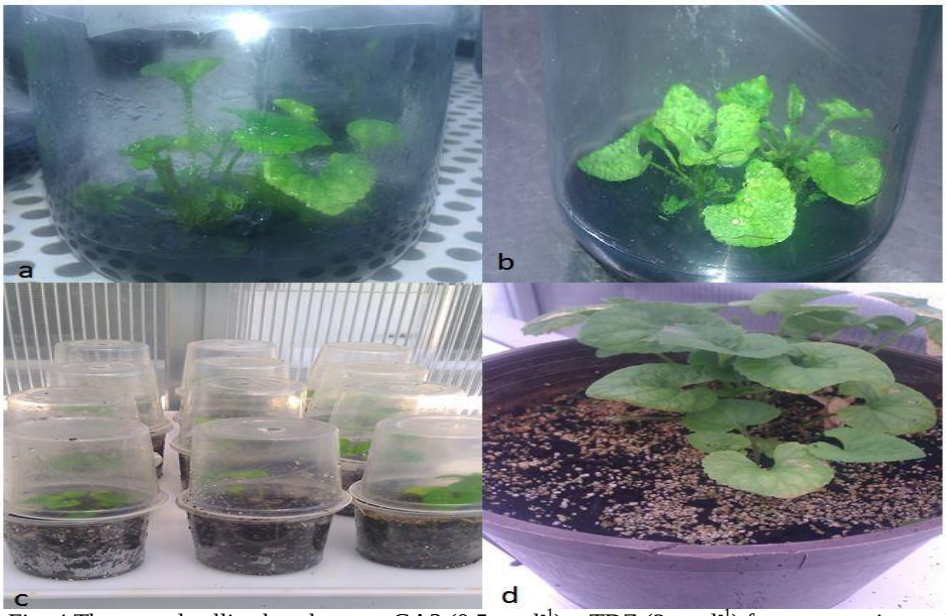


Fig. 4.The rooted calli sub-culture on GA3 (0.5 $mg.l^{-1}$) + TDZ (2 $mg.l^{-1}$) for regeneration and further shoot multiplication (a and b); hardened plantlets under greenhouse condition (c & d).

Table 2. The effects of TDZ and GA3 on regeneration percentage, mean number and length of callus derived micro- shoots of *V. odorata*.

Plant Growth Regulators ($mg.l^{-1}$)	% Regeneration	shoot number	Shoot length
GA3 (0) + TDZ (0), Control	0.00^{e}	0.00^{d}	0.00^{f}
GA3 (0.5) + TDZ (0)	0.00^{e}	0.00^{d}	0.00^{f}
GA3 (1) + TDZ (0)	0.00^{e}	0.00^{d}	0.00^{f}
GA3 (1.5) + TDZ (0)	0.00^{e}	0.00^{d}	0.00^{f}
GA3 (2) + TDZ (0)	0.00^{e}	0.00^{d}	0.00^{f}
GA3 (0) + TDZ (1)	0.00^{e}	0.00^{d}	0.00^{f}
GA3 (0.5) + TDZ (1)	0.00^{e}	0.00^{d}	0.00^{f}
GA3 (1) + TDZ (1)	0.00^{e}	0.00^{d}	0.00^{f}
GA3 (1.5) + TDZ (1)	0.00^{e}	0.00^{d}	0.00^{f}
GA3 (2) + TDZ (1)	0.00^{e}	0.00^{d}	0.00^{f}
GA3 (0) + TDZ (2)	30.00^{d}	2.15^{d}	3.35^{e}
GA3 (0.5) + TDZ (2)	78.33^{a}	6.58^{a}	7.66^{a}
GA3 (1) + TDZ (2)	76.66^{a}	6.58^{a}	6.91^{b}
GA3 (1.5) + TDZ (2)	63.33^{b}	5.16^{b}	5.50^{d}
GA3 (2) + TDZ (2)	60.00^{b}	5.00^{bc}	5.00^{d}
GA3 (0) + TDZ (3)	28.00^{d}	2.00^{d}	3.12^{e}
GA3 (0.5) + TDZ (3)	69.16^{b}	5.91^{b}	7.16^{c}
GA3 (1) + TDZ (3)	67.50^{b}	5.91^{b}	7.25^{c}
GA3 (1.5) + TDZ (3)	61.66^{b}	4.03^{c}	5.40^{d}
GA3 (2) + TDZ (3)	42.00^{c}	2.15^{d}	5.80^{d}
GA3 (0) + TDZ (4)	0.00^{e}	0.00^{d}	0.00^{f}
GA3 (0.5) + TDZ (4)	0.00^{e}	0.00^{d}	0.00^{f}
GA3 (1) + TDZ (4)	0.00^{e}	0.00^{d}	0.00^{f}
GA3 (1.5) + TDZ (4)	0.00^{e}	0.00^{d}	0.00^{f}
GA3 (2) + TDZ (4)	0.00^{e}	0.00^{d}	0.00^{f}

Means followed by different letters are significantly different at ***p = 0.05*** *according to LSD test.*

Maximum mean number of shoots (6.58, Figure 4 b) was observed in MS media supplemented with TDZ (2 $mg.l^{-1}$) combined with GA3 (0.5 or 1$mg.l^{-1}$).TDZ is a synthetic phenyl urea cytokinin like compound that has been proved to be highly effective regulator of shoot morphogenesis. It is also effective in shoot regeneration in many recalcitrant species (Huttenman, 1993). Dabauza et al., (2001) combined GA3 with BA, IAA and thidiazuron (TDZ), respectively. When TDZ was combined with GA3, explants differentiated at highest frequencies. Gibberellins are a naturally occurring plant hormone that affects cell enlargement and division which leads to internodes elongation in stems (Jatoi, 1999). GA3 can be stimulating shoot elongation by inhibiting the action of auxins in meristematic regions (Gaspar, 1996). The highest length of shoots (7.66) was obtained in the MS media having GA3 (0.5 $mg.l^{-1}$) + TDZ (2 $mg.l^{-1}$). This result is an agreement with the available reports for *Viola odorata* (Naeem et al., 2013) and *Viola wttirockinii* (Wang at al., 2006) in which shooting was occurred on MS media containing TDZ and AgNO3 with plant growth regulators GA3 and NAA. MSmediumcontaining1 $mg.l^{-1}$ GA3 produced maximum of shoot elongation in direct and indirect regenerated shoots from epicotyls explants of *Withania somnifera* (Udayakumar et al., 2013).

Hardening. For acclimation of plantlets, regenerated plantlets with enough roots were transferred to greenhouse. Results showed the plantlets excellently adapted to greenhouse condition (Figure 4, c and d). In the present study, the effect of various plant growth regulators and three kinds of explants on callus induction, regeneration and rooting of plantlets was determined and an efficient protocol for tissue culture of *Viola odorata* was set up. Results showed the combination of 2, 4-D and NAA was effective in callus induction, and can get enough calluses for micro-propagation of this medicinal important plant. The explants were also important for callus induction, regeneration and rooting of plantlets, and best results were obtained when leaf pieces were used as explants. Also our results have shown that shoot multiplication and length of branches were affected by the GA3 and TDZ.

CONCLUSIONS

Viola odorata is a hardy herbaceous flowering perennial medicinal plant mainly used as an herbal cure in the diabetes and cancer. Because of its hard seed coat and thermal dormancy and extensive use in herbal formulation without commercial cultivation became rare. Followed our previous report on seed germination, the present study introduced the useful protocol for callus induction and shoot regeneration for conservation purpose and supplying the raw plant materials required by developing pharmacological industry in future.

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