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Direct organogenesis from leaf and internode explants of in vitro raised wintergreen plant (*Gaultheria fragrantissima*)

R.A. Ranyaphia[a,*], **A.A. Maoa**[a], **S.K. Borthakurb**[b]

[a] Plant Tissue Culture Laboratory, Botanical Survey of India, Eastern Regional Centre, Meghalaya-793003, India
[b] Department of Botany, Gauhati University, Assam, India

*Corresponding author, e-mail: ranyavahum@gmail.com

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ABSTRACT: Protocol for rapid regeneration of the important medicinal plant *Gaultheria fragrantissima* Wall. (Ericaceae) through direct organogenesis from leaf, internode (stem without axillary bud), and root explants of in vitro raised seedlings has been developed. Different cytokinins (thidiazuron, 6-benzyladenine, kinetin, and 2-isopentenyl adenine) at a range of various concentrations in modified rhododendron medium have been investigated for organogenesis. Among the four cytokinin investigated, only thidiazuron (TDZ) was effective for direct shoot regeneration from leaf and internode explants but root explants failed to respond. Percentage of response in shoot bud induction increases as the concentration of TDZ increases. After 6 weeks culture on TDZ supplemented medium the shoots were elongated on medium devoid of TDZ. Although the percentage of response was larger at higher concentration of TDZ, 1–2 mg/l TDZ was the optimum concentration for obtaining normal, healthy shoots. The shoots developed from shoot bud induced at higher concentration of TDZ were stunted and hyperhydric. Explant culture on induction medium containing TDZ for more than 6 weeks has a detrimental effect on shoot development. Supplement of 2iP (5, 8 mg/l) or Kin (4, 6 mg/l) on the development medium enhanced the number of shoot and shoot length. Half strength modified rhododendron medium supplemented with 2iP (5 mg/l) was the ideal shoot development medium, producing 12–16 shoots from single explant within 8 weeks. Significant carry over effect of TDZ on root inhibition of microshoots was observed. Two month old rooted plantlets were transferred from lab to land and successfully established.

KEYWORDS: *Gaultheria fragrantissima*, regeneration, TDZ

INTRODUCTION

Gaultheria fragrantissima Wall. (Ericaceae), commonly known as wintergreen, is a bushy evergreen shrub of higher elevation growing in shaded woodland and margin of forests[1]. The plant grows on sandy (light), loamy (medium), and acidic soil. This aromatic plant has high content of essential oil rich in methyl salicylate. The oil is extracted by distillation of leaves. The oil has high demand in pharmaceutical and food industries. It has been used as antiseptic, carminative, rheumatic and arthritis treatment[2,3], flavouring agent (confectioneries, herbal tea, toothpaste, etc.) and condiment[4]. Methyl salicylates are the natural precursors of pharmaceutical aspirin. In the US there are more than 40 products that contain methyl salicylate as an active ingredient to treat various kinds of external pains[5]. The yield of wintergreen oil from the plants of north east India is reported to be higher than the other parts of India[6,7].

A standardized protocol for clonal micropropagation of *G. fragrantissima* from axillary shoot meristem was developed[8]. However, there is no report of direct or indirect organogenesis of this species. Regeneration of plantlets from explants such a root, leaf, and internode segment would permit genetic manipulation of *G. fragrantissima*. An indirect shoot regeneration system can be used to identify or induce somaclonal variant and to develop transgenic plant following genetic transformation of plant cell, whereas direct adventitious shoot formation without the intervention of callus is a reliable procedure for clonal propagation as it prevents somaclonal variations in the cultures. Hence the objective of the present study was to determine the optimal condition for in vitro plant regeneration via direct organogenesis using thidiazuron (TDZ), a substituted phenyl urea compound having cytokinin and auxin like effect[9], reported TDZ as the most

Table 1 Constituents of MRM for direct shoot regeneration of *G. fragrantissima*.

Constituents (mg/l)	SI[a]	SD[b]
Ammonium nitrate (NH_4NO_3)	400.0	200.0
Potassium nitrate (KNO_3)	480.0	240.0
Sodium dihydrogen orthophosphate ($NaH_2PO_4 \cdot 2\,H_2O$)	380.0	190.0
Calcium chloride ($CaCl_2 \cdot 2\,H_{20}$)	440	220.0
Magnesium sulphate ($MgSO_4 \cdot 7\,H_2O$)	370	185.0
Ferrous sulphate ($FeSO_4 \cdot 7\,H_2O$)	27.8	13.9
Na_2-EDTA	37.3	18.65
Boric acid (H_3BO_3)	6.2	3.1
Manganese sulphate ($MnO_4 \cdot H_2O$)	16.9	8.45
Zinc sulphate ($ZnO_4 \cdot 7\,H_2O$)	8.6	4.3
Sodium molybdate ($NaMoO_4 \cdot 2\,H_2O$)	0.25	0.125
Copper sulphate ($CuO_4 \cdot 5\,H_2O$)	0.025	0.0125
Cobalt chloride ($CoCl_2 \cdot 5\,H_2O$)	0.025	0.0125
Inositol	1.00	0.5
Thiamine HCl	0.4	0.2

[a] Shoot induction from leaf and internode.
[b] Shoot development and rooting.

potent of the diphenyl ureas evaluated for use in plant tissue culture [10]. TDZ has proved to be very effective in inducing in vitro shoot regeneration of many woody plants [11] including species from Ericaceae family such as lingonberry [12] and blueberry [13]. The effectiveness of TDZ on shoot regeneration in vitro has not been studied until date with *G. fragrantissima*. Hence in the present study, the efficacy of TDZ and other cytokinin namely benzyladenine (BA), 2-isopentenyl adenine (2ip), and kinetin (Kin) for shoot regeneration via direct organogenesis was investigated for rapid plant regeneration.

MATERIALS AND METHODS

The matured fruits (drupe) were collected in the months of July–August from the natural habitat, Shillong Peak, Meghalaya at an altitude of 1830 m. The seeds were removed from the fruit and treated with 10% (v/v) sodium hypochlorite solution with 1–2 drops of Tween 20 for 40 min followed by 3 washes with sterile distilled water under aseptic condition. The seeds were germinated on modified basal rhododendron medium (MRM) [14]. Rhododendron medium [14] was modified by reducing the iron source, $FeSO_4$-EDTA to half of its original content as standardized for *G. fragrantissima* culture by the same authors. The constituent of the medium is given in Table 1.

For direct adventitious shoot regeneration, leaf (0.5 cm^2), internode, and root segments of about 0.5–1 cm from 6 month old in vitro raised seedlings were used as explants. Two explants were inoculated in each test tube containing MRM supplemented with TDZ (0.5, 1, 2, 3, and 4 mg/l), BA (2, 5, 10 mg/l), Kin (2, 5, 10 mg/l), and 2iP (5, 10, 15 mg/l) separately and in combination with indole-3-acetic acid (IAA) and 1-naphthaleneacetic acid (NAA) (1, 2 mg/l). MRM without any growth regulator was used as control in all the experiments.

After 6 weeks culture of explant on the induction medium containing TDZ, the shoot bud induced on different concentration of TDZ were segregated and subcultured on full strength or half strength MRM devoid of TDZ for shoot development. The effect of culture period on TDZ containing medium was also studied by subculturing the shoot bud on half strength basal medium after 5, 6, and 7 weeks culture. Thereafter, all the shoot elongation experiments were studied on half strength medium after 6 weeks culture on induction medium.

The effect of 2ip and Kin on shoot development was studied by a subculture of shoot bud induced by (2 mg/l) TDZ on half strength medium containing 2iP (2, 5, 8 mg/l) and Kin (2, 4, 6 mg/l). To study the carry over effect of TDZ on rooting, the shoots induced on different concentration of TDZ were segregated according to the concentration of TDZ used in the induction medium and cultured separately. After 8 weeks culture on development medium, 2–3 cm long shoots having 3–5 leaves were cultured on half strength medium supplemented with Indole 3-butyric acid (IBA) (1 mg/l) for rooting. Two month old rooted plantlet were taken out from the culture vessel, wash the adhering medium away, treated with fungicide (Bavistin, 0.4% w/v) for 15 min, and planted on coarse sand. The plantlets were irrigated with half strength MRM salt solution every week.

Culture condition

Basal medium contains sucrose (3%, w/v), activated charcoal (0.1%, w/v) and Difco-Bacto agar (0.8%, w/v). The pH of the medium was adjusted to 5.8 before autoclaving. Aliquots of medium were dispensed in conical flasks or test tubes, cap with aluminium foil, and, along with all the equipments needed for the inoculation, autoclaved at 121 °C and 15 psi for 20 min. All the culture was maintained under warm white fluorescent light at an irradiance of 50 mmol m^{-2} s^{-1}, with a 16 h photoperiod and at 24 ± 2 °C.

Experimental design and measurement

For shoot bud induction on TDZ, sixty explants were used for each treatment. Each treatment was repeated

four times by using a completely randomized design. Number of days taken for shoot bud induction was noted. The percentage of explants responded in shoot bud induction was recorded after 5 weeks. Ten explants each with the shoot bud induced on different concentrations of TDZ medium were separately transferred to shoot development medium. After 4 and 8 weeks in the shoot development medium, the numbers of shoots per explant and shoot lengths were recorded. For rooting, number of days taken for root initiation was noted, and the percentage of rooted plantlet, root number, and length were calculated after 8 weeks of culture. Collected data were subjected to ANOVA and the treatment means were compared by least significant difference (LSD) at 5% level of significance.

RESULTS AND DISCUSSION

Seeds imbibe and swell within a week and germination starts in 15–20 days, 79–86% of seeds germinated by 6 weeks. Leaf and internode explants cultured on medium with TDZ showed gradual swelling after a week. Direct shoot bud protuberance was observed from the cut ends of leaf and internode explants within 5 weeks. Shoot regenerates directly without the intervention of callus formation. The percentage of response (62–96%) in shoot bud induction increases as the concentration of TDZ increases from both the explants (Table 2). The percentage of response was slightly higher from a leaf explant than the internode explants. There was no response in shoot bud induction from root explant.

In the basal medium and other cytokinin treatments, there was no response to organogenesis, rather the explants turned brown and ultimately died within 5 weeks. As with our result, *Filipendula ulmaria* adventitious shoot buds were induced by TDZ, but not by other cytokinins[15]. Variation in the activity of different cytokinins can be explained by their differential uptake rate reported in different genomes[16], varied translocation rates to meristematic regions, and metabolic processes, in which the cytokinin may be degraded or conjugated with sugars or amino acids to form biologically inert compounds as earlier reported[17,18].

TDZ, when used in combination with auxins, drastically reduced the percentage of response (Table 2). Moreover there was no significant increase in the number of shoots regeneration per explant. In contrast, Yildirim and Turker[15] observed a significant increase in the percentage of explants forming shoot and the mean shoot number per explant when TDZ was used in combination with IAA.

Table 2 Percentage of response in direct shoot regeneration of leaf and internode explants to direct organogenesis after 5 weeks culture on induction medium containing different concentrations of TDZ alone or in combination with auxin.

PGRs (mg/l)			Response (%)	
TDZ	NAA	IAA	Leaf	Internode
0	0	0	0	0
0.5			67^{e}	62^{e}
1			78^{d}	73^{cd}
2			86^{c}	78^{c}
3			93^{ab}	87^{ab}
4			96^{a}	91^{a}
2	1		57^{fghijk}	55^{ghi}
2	2		52^{jkl}	54^{ghij}
3	1		62^{efgh}	59^{efg}
3	2		65^{ef}	53^{hij}
2		1	61^{fghi}	58^{efgh}
2		2	55^{ijk}	54^{ghij}
3		1	63^{efg}	61^{ef}
3		2	58^{fghij}	57^{efghi}
SE			± 3.4	± 3.1
LSD			5.6	5.2

Comparison within the columns: Mean followed by common letter are not significantly different ($P < 0.05$).

Multiple shoot buds occurred as tight bud clusters. After a period of 5 weeks, a thick mat of regenerating shoot buds covered the explant. There is no distinct shoot elongation, making it difficult to count the number of shoot buds per explant. A similar pattern of shoot bud development from leaf explants was reported by Banerjee et al[19]. Although it was difficult to count the number of shoot buds, it was clearly visible that the number of shoot buds increases with every increase in TDZ concentration. In lower concentrations of TDZ the shoots elongated with some expanded leaves, whereas in the higher concentration the shoot buds were highly vitrified and fail to elongate.

The efficacy of TDZ for induction of direct shoot organogenesis is well documented in several woody plants[20–22]. Moreover some researchers[23,24] have reported TDZ to be an essential growth regulator for shoot induction from leaf explant of Ericaceae family.

It was emphasized that the efficiency of TDZ may be due to its ability to induce cytokinin accumulation[25] or enhance the accumulation and translocation of auxin within the tissue[26]. TDZ is also suspected of promoting regulated morphogenesis in plants through the modulation of endogenous cytokinin and auxin[27]. Heutteman and Preece[11] and De Gyves et al[28] em-

Table 3 Carry over effect of TDZ on number of shoots after transfer on shoot development medium (full and half strength basal MRM). Observation after 4 and 8 weeks culture.

Induction	Leaf explant				Internode explant			
medium	Shoot development medium (MRM)							
containing	Full strength		Half strength		Full strength		Half strength	
TDZ (mg/l)	4 week	8 week	4 week	8 week	4 week	8 week	4 week	8 week
0.5	0.8^{d}	1.8^{d}	1.7^{d}	3.1^{d}	1.4^{de}	2.8^{de}	2.0^{d}	4.3^{d}
1	1.6^{c}	3.7^{bc}	2.7^{bc}	5.2^{bc}	2.3^{c}	4.7^{abc}	3.2^{abc}	6.5^{bc}
2	2.6^{ab}	5.3^{ab}	3.4^{ab}	6.8^{ab}	3.4^{ab}	5.9^{ab}	4.1^{ab}	8.3^{ab}
3	3.1^{a}	5.8^{a}	3.6^{a}	7.2^{a}	3.7^{a}	6.2^{a}	4.4^{a}	8.9^{a}
4	0.7^{de}	1.6^{de}	1.4^{de}	2.8^{de}	1.6^{cd}	3.4^{cd}	2.2^{cd}	4.1^{de}
SE	± 0.27	± 0.48	± 0.34	± 0.54	± 0.36	± 0.52	± 0.43	± 0.58
LSD	0.8	1.7	0.9	1.9	0.9	1.8	1.1	2.1

Comparison within the columns: Mean values followed by common letter are not significantly different ($P < 0.05$).

Table 4 Carry over effect of TDZ on shoot length (cm) produced after transfer on shoot development medium (full and half strength basal MRM). Observation after 4 and 8 weeks culture.

Induction	Leaf explant				Internode explant			
medium	Shoot development medium (MRM)							
containing	Full strength		Half strength		Full strength		Half strength	
TDZ (mg/l)	4 week	8 week	4 week	8 week	4 week	8 week	4 week	8 week
0.5	0.19^{ab}	0.37^{bc}	0.31^{ab}	0.62^{ab}	0.24^{ab}	0.52^{ab}	0.34^{ab}	0.68^{ab}
1	0.21^{a}	0.48^{a}	0.33^{a}	0.69^{a}	0.29^{a}	0.59^{a}	0.38^{a}	0.76^{a}
2	0.17^{abc}	0.42^{ab}	0.26^{abc}	0.51^{bc}	0.18^{c}	0.37^{c}	0.31^{abc}	0.63^{bc}
3	0.12^{cd}	0.23^{d}	0.22^{cd}	0.43^{cd}	0.15^{cd}	0.34^{cd}	0.27^{bcd}	0.52^{d}
4	0.06^{de}	0.13^{de}	0.11^{e}	0.17^{e}	0.1^{e}	0.18^{e}	0.15^{e}	0.28^{e}
SE	± 0.04	± 0.07	± 0.05	± 0.08	± 0.05	± 0.08	± 0.06	± 0.09
LSD	0.07	0.11	0.09	0.12	0.08	0.12	0.09	0.13

Comparison within the columns: Mean values followed by common letter are not significantly different ($P < 0.05$).

phasized the potential use of TDZ in the regulation of adventitious shoot production and hypothesize on the synergism existing between TDZ and both endogenous and exogenous auxin.

CARRY OVER EFFECT OF TDZ ON SHOOT DEVELOPMENT

The number of shoots produced increases with each increase in the concentration of TDZ with maximum number of shoot developed on 2 and 3 mg/l TDZ from leaf and stem explant (Table 3). Thereafter, a sharp decrease in the number of shoots per explant was evident at 4 mg/l TDZ after 8 weeks culture. At this concentration only few shoots developed and more of highly vitrified leafy structure continues to proliferate.

TDZ concentration has a significant effect on shoot length as well; shoots derived from the lower concentration of TDZ (0.5, 1, 2 mg/l) were significantly longer (Table 4). Shoot length significantly reduces with TDZ concentration higher than 2 mg/l. Moreover, shoot vigour decreases with increasing concentration of TDZ. At higher concentrations (3 and 4 mg/l) of TDZ the shoot produced were vitrified and stunted. Hence the optimum concentration of TDZ for direct shoot bud induction in *G. fragrantissima* is 1–2 mg/l TDZ, considering both the number of shoots developed and shoot length. Although the shoot regeneration was more at higher concentration of TDZ, the shoots were abnormal, stunted and hyperhydric. Whereas shoot induced on lower concentrations of TDZ (0.5–2 mg/l) can be easily elongated when cultured on development medium. Similarly report of 1 mg/l TDZ as the optimum concentration for shoot organogenesis from leaf explant in *Rehmannia*

Table 5 Effect of explant type and medium strength on number of shoot development. Observation after 8 weeks culture.

Explant type	Shoot development medium (MRM)	Induction medium containing TDZ(mg/l)				
		0.5	1	2	3	4
Internode	Full strength	2.8^{bc}	4.7^{bc}	5.9^{bc}	6.2^{bc}	3.4^{ab}
	Half strength	4.3^{a}	6.5^{a}	8.3^{a}	8.9^{a}	4.1^{a}
Leaf	Full strength	1.8^{cd}	3.7^{cd}	5.3^{cd}	5.8^{cd}	1.6^{d}
	Half strength	3.1^{b}	5.2^{b}	6.8^{b}	7.2^{b}	2.8^{bc}
SE		± 0.53	± 0.57	± 0.62	± 0.61	± 0.58
LSD		1.1	1.3	1.5	1.4	1.2

Comparison within the columns: Mean values followed by common letter are not significantly different ($P < 0.05$).

Table 6 Effect of explant type and medium strength on shoot length (cm). Observation after 8 weeks culture.

Explant type	Shoot development medium (MRM)	Induction medium containing TDZ(mg/l)				
		0.5	1	2	3	4
Internode	Full strength	0.52^{c}	0.59^{c}	0.37^{d}	0.34^{c}	0.18^{c}
	Half strength	0.68^{a}	0.76^{a}	0.63^{a}	0.52^{a}	0.28^{a}
Leaf	Full strength	0.37^{d}	0.48^{d}	0.42^{c}	0.23^{d}	0.13^{d}
	Half strength	0.62^{b}	0.69^{b}	0.51^{b}	0.43^{b}	0.17^{b}
SE		± 0.04	± 0.03	± 0.03	± 0.03	± 0.02
LSD		0.06	0.05	0.05	0.04	0.04

Comparison within the columns: Mean values followed by common letter are not significantly different ($P < 0.05$).

glutinosa was reported[29].

Production of short and vitrified shoots on TDZ medium was earlier reported[11,30]. As with our finding, difficulty in shoot development and elongation despite the multiple shoot bud induction at higher concentration of TDZ was reported in *Capsicum annum*[31] and *Rubus* cultivars[20].

Of the different strength of medium studied for shoot development, the number of shoots as well as shoot length was significantly higher on half strength medium than full strength medium (Tables 5 and 6). Similarly, better shoot elongation of multiple shoots induced from axillary meristem of *G. fragrantissima* was observed on half strength medium rather than full strength medium[8].

In addition to the effect of TDZ concentration on shoot development, the duration of culture on TDZ containing medium also has a significant effect on the shoot development. The number of shoots developed from explants cultured on TDZ containing medium for more than 6 weeks drastically reduced (Tables 7 and 8). When the explants were cultured on TDZ containing medium for more than 7 weeks, the shoot buds became vitrified and difficult to develop to healthy shoot. However there was no significant difference in the number of shoots developed from explants exposed to TDZ for 5 and 6 weeks. Hence, 5–6 weeks culture on shoot induction medium containing TDZ is optimum for obtaining maximum, healthy shoots. Similar report on the effect of exposure duration to TDZ was earlier reported[9]. According to their finding, 4 day exposure to 5.0 µM TDZ resulted in the highest degree of somatic embryo formation in *Geranium*.

EFFECT OF 2IP AND KIN ON SHOOT ELONGATION

Both 2iP and Kin had a significant effect on the shoot development of the shoot bud produced on TDZ. The number of shoots developed on medium containing 2ip (5, 8 mg/l) or Kin (5 mg/l) was significantly higher than the number of shoots developed on control (Table 9). Similarly, shoots developed on 2ip (5 mg/l) or Kin (4 and 6 mg/l) supplemented medium were significantly longer than those shoots developed on control (Table 10). Medium containing 2iP (5 mg/l) produced highest number of shoots with longer shoot length and hence the optimum medium for shoot development was half strength MRM supplemented with 2iP (5 mg/l). Based on our finding in *G. fragrantissima*, a secondary medium for shoot development is essential for successful elongation of shoot buds induced on TDZ as earlier reported in apple[18]. Development of two step shoot regeneration for other Ericaceae family is also reported[13,32,33].

Table 7 Effect of exposure period (week) to TDZ on number of shoot development. Observation after 8 weeks culture.

Explant type	Culture period (weeks)	Induction medium containing TDZ (mg/l)				
		0.5	1	2	3	4
internode	5	3.7^{ab}	5.4^{ab}	7.2^{ab}	7.6^{ab}	3.3^{ab}
	6	4.3^{a}	6.5^{a}	8.3^{a}	8.9^{a}	4.1^{a}
	7	3.1^{bc}	3.9^{c}	4.6^{c}	4.8^{c}	2.3^{bcd}
leaf	5	2.2^{c}	3.7^{d}	4.3^{cd}	4.7^{cd}	2.2^{bcde}
	6	3.1^{bc}	5.2^{abc}	6.8^{b}	7.2^{b}	2.8^{bc}
	7	1.6^{cd}	2.4^{de}	3.7^{cde}	3.6^{cde}	1.5^{de}
SE		± 0.6	± 0.8	± 0.8	± 0.7	± 0.6
LSD		1.2	1.4	1.5	1.6	1.3

Comparison within the columns: Mean values followed by common letter are not significantly different ($P < 0.05$).

Table 8 Effect of exposure period (weeks) to TDZ on shoot length (cm). Observation after 8 weeks culture.

Explant type	Culture period (weeks)	Induction medium containing TDZ (mg/l)				
		0.5	1	2	3	4
Internode	5	0.71^{a}	0.79^{a}	0.68^{a}	0.57^{a}	0.32^{a}
	6	0.68^{ab}	0.76^{ab}	0.63^{ab}	0.52^{ab}	0.28^{ab}
	7	0.56^{cde}	0.63^{c}	0.51^{cd}	0.41^{cde}	0.17^{cd}
leaf	5	0.67^{abc}	0.71^{abc}	0.54^{c}	0.46^{c}	0.21^{c}
	6	0.62^{abcd}	0.68^{bc}	0.51^{cd}	0.43^{cd}	0.17^{cd}
	7	0.49^{ef}	0.52^{d}	0.40^{e}	0.38^{de}	0.11^{e}
SE		± 0.06	± 0.05	± 0.04	± 0.04	± 0.03
LSD		0.12	0.11	0.08	0.08	0.06

Comparison within the columns: Mean values followed by common letter are not significantly different ($P < 0.05$).

Effect of explant on shoot regeneration and shoot vigour

The type of explant has a great effect on the organogenesis and growth of the regenerated shoot. Comparing the two explants namely leaf and internode in the present study, internode was found to be superior to leaf explants. The percentage of response to shoot regeneration was higher from leaf explants (Table 2) however; internode explants produced larger number of shoots per explant (Tables 5 and 7) and longer shoots (Tables 6 and 8) on the same treatment. The shoots produced from internode explant had vigorous growth but the shoots derived from leaf explant were vitrified and stunted in growth. Ali and Mirza[33] had also reported stem as the ideal explant for shoot regeneration. In contrast to our observation, there are reports[34,35] that regeneration frequency was higher with leaf explants and the shoot derived from leaf explant was more vigorous. The differential response of these two explants may be attributed to the difference in anatomical structure[36], differential reactivity of the explant to media component[37], and the interaction between the type of tissue and the concentration of endogenous growth regulators[38].

Carry over effect of TDZ on in vitro rooting

There are reports of repression of root formation by using excess cytokinin in the propagation medium[39,40]. Likewise in the present study, TDZ has a considerable effect on root initiation, percentage of rooted shoot, as well as number of root and root length (Table 11). The percentage of rooting was significantly different between the shoot derived from control and TDZ supplemented medium. The shoots from control initiated root within 3 weeks followed by shoot obtained from 0.5 and 1 mg/l TDZ in 4 weeks and 5 weeks for shoots obtained from 2 and 3 mg/l TDZ.

Number of roots and root length were not significantly different between control and 0.5 mg/l TDZ. Also, there was no significant difference in the percentage of rooting, root number, and length between 0.5 and 1 mg/l TDZ. However, in concentrations higher than 1 mg/l the percentage of rooting, number of root produced, as well as root length drastically reduced with significant difference from the other treat-

Table 9 Effect of cytokinin on number of shoot development from shoot bud induced on TDZ (2 mg/l). Observation after 4 and 8 weeks culture.

Treatment		Internode explant		Leaf explant	
PGRs	(mg/l)	4 week	8 week	4 week	8 week
Control	0	4.2^{efg}	8.3^{fg}	3.3^{ef}	6.8^{de}
2iP	2	5.2^{e}	11.9^{bcde}	5.2^{bcd}	9.5^{cd}
	5	8.3^{a}	15.6^{a}	6.7^{a}	12.6^{a}
	8	7.2^{b}	13.7^{ab}	5.4^{bc}	10.2^{bcd}
Kin	2	4.9^{ef}	10.1^{ef}	3.8^{e}	8.2^{d}
	4	6.7^{bc}	13.5^{abc}	5.6^{b}	11.5^{ab}
	6	6.4^{bcd}	13.1^{bcd}	5.4^{bc}	11.2^{abc}
SE		± 0.39	± 0.48	± 0.33	± 0.42
LSD		1.1	2.3	1.1	1.8

Comparison within the columns: Mean values followed by common letter are not significantly different ($P < 0.05$).

Table 10 Effect of cytokinin on shoot length (cm) of shoot bud induced on TDZ (2 mg/l). Observation after 4 and 8 weeks culture.

Treatment		Internode explant		Leaf explant	
PGRs	(mg/l)	4 week	8 week	4 week	8 week
Control	0	0.31^{cde}	0.59^{de}	0.17^{f}	0.35^{f}
2iP	2	0.36^{abc}	0.68^{bc}	0.29^{bc}	0.52^{de}
	5	0.42^{a}	0.81^{a}	0.37^{a}	0.74^{a}
	8	0.34^{bcd}	0.65^{bcd}	0.28^{d}	0.57^{cd}
Kin	2	0.32^{cd}	0.62^{cde}	0.26^{de}	0.52^{de}
	4	0.38^{ab}	0.73^{abc}	0.36^{ab}	0.71^{ab}
	6	0.36^{abc}	0.74^{ab}	0.34^{abc}	0.68^{abc}
SE		± 0.05	± 0.09	± 0.05	± 0.08
LSD		0.08	0.12	0.08	0.12

Comparison within the columns: Mean values followed by common letter are not significantly different ($P < 0.05$).

ments. The observation is similar to earlier reports that TDZ has inhibition effect on rooting [11,30]. Most of the shoots without roots were the vitrified shoots obtained from the higher TDZ treatment. However, many vitrified shoots slowly lost the vitreous condition in subsequent subcultures and the shoot length increased considerably and successfully rooted.

Eighty percent of plantlets survived and established within 6 weeks. After 3 months growth in the greenhouse the plantlets were transferred to pot containing soil and leaf mould (1:1) to hasten the growth or transfer to the field directly.

From the present investigation, the protocol for direct shoot regeneration can be summarized that MRM is the suitable media composition and internode explant is the ideal explant for direct shoot regeneration of *G. fragrantissima*. For direct shoot induction, explant was cultured on TDZ (1–2 mg/l) containing medium for 5–6 weeks. Thereafter, the shoot buds were transferred to half strength medium devoid on TDZ for shoot development. To obtain more shoots with vigorous growth, 2iP should be included in the shoot development medium. After 8 weeks culture on development medium, longer shoots were excised out and culture on half strength medium containing 1 mg/l IBA for rooting of the shoots in vitro. The smaller shoots were subcultured on fresh medium. 8 weeks old rooted plantlets were acclimatized on sand in greenhouse condition. After 3 months the plantlets were ready to transfer to field. The plants did not show any morphological abnormalities when compared with donor plants.

Table 11 Carry over effect of cytokinin on in vitro rooting. Observation after 8 weeks culture.

TDZ on induction medium (mg/l)	Rooting (%)	Root number	Root length (cm)
0	87^{a}	8.6^{a}	2.3^{a}
0.5	81^{b}	8.2^{ab}	2.1^{ab}
1	72^{c}	7.4^{bc}	1.7^{bc}
2	61^{d}	6.1^{d}	1.2^{d}
3	48^{e}	4.8^{e}	0.7^{e}
SE	± 0.34	± 0.65	± 0.3
LSD	5.2	1.2	0.5

Comparison within the columns: Mean values followed by common letter are not significantly different ($P < 0.05$).

The protocol standardized for adventitious shoot regeneration from leaf and internode (stem) segment of *Gaultheria fragrantissima* will be a good alternative of in vitro propagation when the number of organized explant is limited. It also paves the way for an opportunity to regenerate plant from tissue manipulated through biotechnology. Many methods available for transformation ultimately end with tissue culture technique emphasizing the need of a well established regeneration system. Hence the present protocol may be useful for improving the oil yield by genetic engineering of the plant in the near future.

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