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Direct and Indirect Somatic Embryogenesis from Petiole and Leaf Explants of Purple Fan Flower (*Scaevola aemula* R. Br. cv. 'Purple Fanfare')

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Abstract**—**Direct and indirect somatic embryogenesis (SE) from petiole and leaf explants of *Scaevola aemula* R. Br. cv. 'Purple Fanfare' was achieved. High frequency of somatic embryos was obtained directly from petiole and leaf explants using an inductive plant growth regulator signal thidiazuron (TDZ). Petiole explants were more responsive to SE than leaves. Plants derived from somatic embryos of petiole explants germinated more readily into plants. SE occurred more efficiently in half-strength Murashige and Skoog (MS) medium than in full-strength MS medium. Non-embryogenic callus induced by 2, 4-dichlorophenoxyacetic acid was used to investigate the feasibility of obtaining SE with TDZ as a secondary inductive plant growth regulator (PGR) signal. Non-embryogenic callus of *S. aemula* was able to convert into an "embryogenic competent mode" with PGR signal. Protocol developed for induction of direct and indirect somatic embryogenesis in S. aemula can improve the large scale propagation system of the plant in future.

Keywords**—**Petiole and leaf explants, *Scaevola aemula*, Somatic embryogenesis

I. INTRODUCTION

BIOTECHNOLOGY is considered as one of the most important dynamic tools in the development of sustainable agriculture. Somatic embryogenesis (SE) is one of the techniques that emerged in recent years with the potential of providing an efficient clonal propagation system. SE can be a very efficient propagation method, if developed with high frequency selections. It has produced up to sixty thousand embryos from a gram of alfalfa leaf tissue [1], [2]. With such advantages, the production of artificial seeds is normally the next step. Furthermore, using this method, somaclonal variation in plants virtually disappears, hence ensuring genetic uniformity in the propagules [3]. This will facilitate at commercial state to produce seeds of hybrids without losing genetic uniformity in the products. The production of artificial seeds can also be very cost effective. In relation to this, SE can be used for mass seed production hence; employment of this method will benefit the seeds industry. The methods developed to desiccate somatic embryos [1], [4] enabled storage and handling analogous to methods used for true seeds.

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The Goodeniaceae family comprises 14 genera and 300 species all endemic to Australia [5]. Certain species of this family is widely used as garden and greenhouse ornamentals; therefore immensely valuable in economic terms. A member of this family, *Scaevola aemula* R. Br. cv. 'Purple Fanfare' (Fig. 1) is one of the most attractive members of the Goodeniaceae which is characterized by fan shaped flowers is endemic to Australia and increasingly being used in ornamental horticulture [5]. *S. aemula* is a hybrid peculiar to Australia and seeds produced through sexual reproduction are not true-to type. The most common method used to propagate this species is by cutting. Cutting propagation is inefficient and labor intensive; therefore clonal propagation methods must be used.

Fig. 1. Flowers of *Scaevola aemula* R. Br. cv. 'Purple Fanfare'.

However, very little research has been conducted to discover the vast opportunities in propagating this species using advanced techniques. In this research, it was expected to exploit direct somatic embryogenesis (DSE) and indirect somatic embryogenesis (ISE) to achieve clonal propagation of Purple Fan Flower (*Scaevola aemula*). The tissues from *Scaevola* have been highly responsive to conventional tissue culture techniques and hence there is a possibility that *Scaevola* would be responsive to the induction process required for somatic embryos to form from a range of somatic tissues [6], [7]. Induction of DSE from leaf explants of *S. aemula* has been reported on MS medium supplemented with 0.2 mg -1 L 2-4-D and 0.2-0.5 mg-1 L 6-benzylaminopurine (BAP) [8], [9].

SE can be classified into three types, DSE, ISE and secondary somatic embryogenesis (SSE). In DSE, somatic embryos are formed directly from the cells of the explants. Most research in the past used DSE as the primary system generally for convenience. However, qualitative and quantitative plant growth regulator (PGR) requirements vary in the induction media depending on the species. The use of auxins and cytokinins in SE are vital as both have their own function as inducers for SE [10]. In ISE, the initial tissues are normally developed as callus and suspension cultures that did not form embryos can be made embryogenic with an appropriate PGRs signals [11].

Auxins have been used as one of the primary chemical inducer of SE as an exogenous signal for various cell divisions vigorously [12]. However, there was a suggestion that cytokinins may also play a key role in SE [13]. Some experiments have employed thidiazuron (TDZ) a phenylurea, which may modulate the action of both auxins and cytokinins relating to SE [14]. A phenylurea, TDZ has been shown in many studies to have a similar mode of action to that of cytokinin [15] and cytokinin and auxin combined action [16]. TDZ (N-phenyl-N'-1, 2, 3-thiadiazol-5-ylurea) is a synthetic phenylurea commonly used as a cotton defoliant and herbicide is now one of the PGRs used in SE [17]. The effectiveness of TDZ has been investigated with many species and the outcomes were promising. One such experiment compared TDZ to a combination of auxin and cytokinin, and the results showed that TDZ alone was superior in inducing SE [18]. Furthermore, it has been reported that TDZ is a very efficient inducer of SE, and only two days on TDZ-supplemented media was sufficient for SEs [19].

The present investigation is a comprehensive study, with major objective of SE in *S. aemula*.

The specific objectives are;

to investigate the embryogenic response of different tissues,

to determine the most effective quality and quantity of auxins and cytokinins in inducing SE,

to evaluate the efficacy of phenylurea TDZ (thidiazuron) in producing somatic embryos in *S. aemula*,

to define the nutritional conditions for efficient somatic embryogenesis and to investigate whether ISE is feasible in *S. aemula*.

This study will undoubtedly lay the foundation for developing commercially feasible large scale propagation system for *S. aemula*.

II. MATERIALS AND METHODS

(a) Direct Somatic Embryogenesis in Scaevola aemula

Plant Material for explant source and culture conditions Sterilized cuttings from cultivated plants of *S. aemula* were made and placed on multiplication media (Half MS [20]) salts, Sucrose at 20 g-1 L, 0.3 μM of BAP (benzylaminopurines), 8 g-1 L of agar, pH at 6.0).

The specimens used in this experiment have been grown under controlled conditions. Five to six cuttings were placed in each jar following 5 weeks of cultivation; the matured plantlets were then sub-cultured into new jars free of any PGRs to prevent any residual effects of BAP affecting the results of the experiments. Two types of explants, leaves and petioles were used. Ten explants per replication, with 3 replications per treatment were prepared. These were placed in 9 cm Petri-dishes with different PGR treatments. The experiment was conducted in two parts: the first part being the induction of somatic embryos through PGR treatments and *in vitro* culturing on media without PGR for embryo development and the second part involved the production of plantlets from the isolated somatic embryos from the preceding experiment.

During the entire *in vitro* culture period the plates and jars were incubated in a tissue culture room at 24 ºC with a photoperiod of 16 hours per day and photosynthetic photon flux density (PPFD) of 30 μM m-2 s-1. Six PGRs were evaluated in this experiment (Table 1).

TABLE 1
PLANT GROWTH REGULATORS AND CONCENTRATION USED FOR THE INDUCTION OF SOMATIC EMBRYOGENESIS IN *SCAEVOLA AEMULA* C.V. 'PURPLE FANFARE'

Treatment No.	Treatments (Concentration in μM)	Treatment No.	Treatments (Concentration in μM)
1	Control	21	5 2,4-D + 5 BAP
2	1 TDZ + 1 IAA	22	5 2,4-D + 1 KINETIN
3	1 TDZ + 5 IAA	23	5 2,4-D + 5 KINETIN
4	1 TDZ + 1 ZEATIN	24	1 IAA
5	1 TDZ + 5 ZEATIN	25	1 IAA + 1 ZEATIN
6	5 TDZ + 1 IAA	26	1 IAA + 5 ZEATIN
7	5 TDZ + 5 IAA	27	1 IAA + 1 BAP
8	5 TDZ + 1 ZEATIN	28	1 IAA + 5 BAP
9	5 TDZ + 5 ZEATIN	29	1 IAA + 1 KINETIN
10	1 2,4-D	30	1 IAA + 5 KINETIN
11	1 2,4-D + 1 ZEATIN	31	5 IAA
12	1 2,4-D + 5 ZEATIN	32	5 IAA + 1 ZEATIN

13	1 2,4-D + 1 BAP	33	5 IAA + 5 ZEATIN
14	1 2,4-D + 5 BAP	34	5 IAA + 1 BAP
15	1 2,4-D + 1 KINETIN	35	5 IAA + 5 BAP
16	1 2,4-D + 5 KINETIN	36	5 IAA + 1 KINETIN
17	5 2,4-D	37	5 IAA + 5 KINETIN
18	5 2,4-D + 1 ZEATIN	38	TDZ-1
19	5 2,4-D + 5 ZEATIN	39	TDZ-5
20	5 2,4-D + 1 BAP		

Production of Plantlets

One somatic embryo from each explant was isolated to evaluate their ability to convert into plantlets. Ten somatic embryos from one replicate of treatment plate were placed in jars with media containing no PGRs for the production of shoots and roots. The evaluation was conducted 5 weeks after somatic embryos have been transferred into jars. Presence of visible shoot and root was considered conversion into plantlets; plantlets were isolated at the end of the five weeks and planted in pre-sterilized soil. Jars with plantlets were first acclimatized by placing in the glasshouse for a week. The plantlets were then washed in water containing a fungicide (0.01% Bentate) to prevent fungal infection and to remove agar. The survival rate was calculated as percentage of surviving plants over the total number of potted plants.

Scoring of the Experiment

Explants were scored from the second week of the experiments. Firstly each plate was scored for percentage of explants with evidence of root hairs. Each of the explants was then scored based on the criteria; Percentage of explants callusing, The amount of callus on a scale of 0-5 ; 0 = zero, 5 = complete callusing of explants, 100%, Percentage of explants exhibiting organogenesis, The mean number of recognizable organs per explants, Percentage of explants with somatic embryo development and The mean number of somatic embryos per explants.

In the second part of the experiment, scoring of isolated somatic embryos were different from above and the following criteria were formulated based on the criteria;
Percentage of somatic embryos producing roots, The mean number of roots per somatic embryo, Percentage of somatic embryos produced shoots, The mean number of shoots produced and The mean length of the shoots.

Statistical Analysis of the Data

Experimental design was Completely Randomized Design (CRD). Data were analyzed for statistical significance, using one-way analysis of variance (ANOVA). Standard errors (S.E.) and protected least squared differences (PLSD) were obtained. All analyses were done using Genstat 4.2, 5th Edition.

(b) Effects of Inorganic Nutrient Levels, Media type, pH and Sugar levels for DSE

Explant preparation and culture methods were similar to previous experiment (Section (a). Only petioles were used as explants. Preparation of treatment plates was similar to the process described in section (a) except the inclusion of variables evaluated in this experiment; quantities of nutrients added were changed and pH was varied and only selected PGRs from previous experiments were included.

PGR treatments, 1 μM of TDZ + 1 μM of IAA and 1 μM of TDZ + 5 μM of IAA were selected based on their superior performance in the previous experiment. Other variables incorporated were;

Half or Full strength MS salts
Sucrose levels of 20, 30 and 40 g-1 L
pH levels of 5.0. 5.5 and 6.0 adjusted prior to autoclaving

The plates were incubated in a tissue culture room at 24 ^{o}C with a photoperiod of 16 hours per day and PPFD of 30 μM m-2 s-1. The basal constituents of the media remained the same as stated in section (a) except for the varied constituents. This part of the experiment consisted of a total number of 36 treatments with 3 replications.

The explants were left on media with PGR for 3 weeks and observations were recorded on a weekly basis. The explants were then transferred to PGR-free plates for a further three weeks before evaluation for embryogenesis. The parameters scored were the same as section (a) and analysis of the data is also similar to that described in section (a).

(c) Indirect Somatic Embryogenesis in Scaevola aemula

Both petioles and leaves were used as explants for the experiment as described in section (a). The steps taken in the procedure is schematically presented in Fig. 2. The whole experiment took 6 weeks to complete but scoring only occurred in the sixth week. The treatment plates were kept in the same place as the two previous experiments (in section (a) and (b)). During the induction period. 2, 4-D was added to half MS media. Two concentrations of 2, 4-D were evaluated; 1 μM and 5 μM (Treatments number 37 and 38 respectively, Table 2). Two explant types (leaf and petiole) were used and 3 replicates were set up. The other components of the media were the same as the previous two experiments (a) and (b). The pH was set at 6.0. After 4 weeks on 2,4-D, one half of the explants were transferred to half MS medium with 0.1% of activated charcoal (denoted by A) and the rest were placed in half MS medium without any PGR (denoted by M). After three days, explants from both pre-treatments were placed on

half MS medium with 5 µM TDZ for induction of somatic embryos. After three days, the explants were transferred to half MS medium. The scoring criteria for this experiment followed the same attributes as in section (a). The scoring was for one week only since the objective was to find evidence of ISE. Statistical analyses were performed as described in section (a).

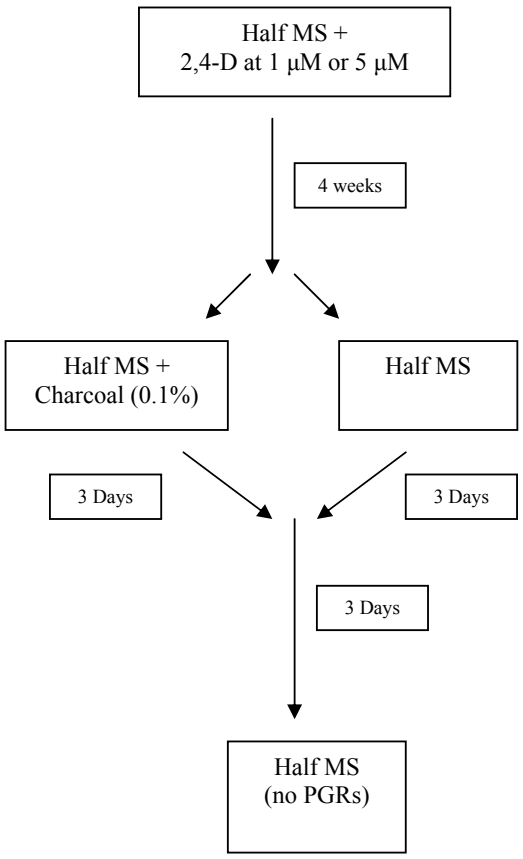

Fig. 2. The summarized protocol for indirect somatic embryogenesis.

TABLE II
TREATMENT AND ASSIGNED TREATMENT NUMBER

Assigned Treatment Number	Concentration of 2,4-D (µM)	Concentration of activated charcoal (%)	Concentration of TDZ (µM)
37A	1	0.1	5
37M	1	0	5
38A	5	0.1	5
38M	5	0	5

III RESULTS

(a) Direct somatic embryogenesis in Scaevola aemula

Production of Somatic Embryos

In the second week, a noticeable increase in callusing was observed except in control but the highest and most consistent increase were from all treatments containing TDZ (>80%) and 2,4-D (>80%). Treatments with IAA had the lowest callusing percentage (33.3%). The embryos produced in this experiment are illustrated in Fig. 3. The calli produced in response to TDZ were green in color, but 2, 4-D induced formation of white and sugary calli on explants (Fig. 3 (A)). The highest % of induction of organogenesis came from treatments containing TDZ and this is also true for the frequency of induction (Table 3). Results indicated that SE occurred only in the treatments that contained TDZ (Fig. 3 (B)). TDZ was the only PGR able to induce SE in both explant types (leaf and petiole) starting from 3rd week (Table 4).

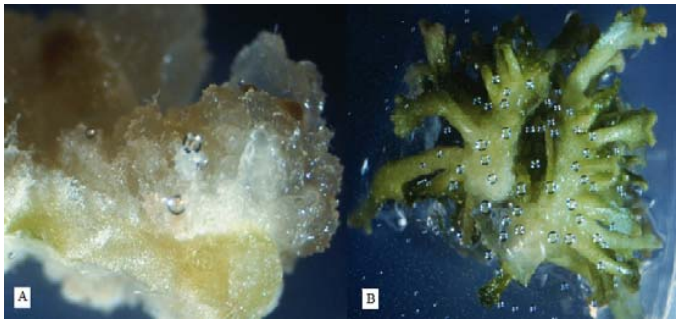

Fig. 3. Micrographs illustrating embryogenic response of explants of *Scaevola aemula* to PGR treatments. (A: The production of white and sugary callus in treatments containing 2,4-D (X 7.5) and B: The high frequency production of somatic embryos in treatments containing TDZ. (X 7.5))

TABLE III
Scoring for percentage of organogenesis and number of organogenic structure formed per leaf and petiole explants over the duration of experiment (only 4th week data are given and the treatment No. 10 to 37 are not included due to no organogenesis response)

Treatment No.	Treatments (concentration in μM)	WEEK 4 - Leaf		WEEK 4 - Petiole	
		Percentage Organogenesis (%)	Number of Organogenic structure/ explant	Percentage Organogenesis (%)	Number of Organogenic structure/ explant
1	Control	0	0	0	0
2	1 TDZ + 1 IAA	100	5.33	96.67	5.57
3	1 TDZ + 5 IAA	100	4.80	100	5.37
4	1 TDZ + 1 ZEATIN	100	5.57	86.67	4.70
5	1 TDZ + 5 ZEATIN	76.67	4.37	96.67	5.20
6	5 TDZ + 1 IAA	96.67	5.80	100	5.53
7	5 TDZ + 5 IAA	96.67	5.46	100	5.13
8	5 TDZ + 1 ZEATIN	100	4.73	96.67	5.50
9	5 TDZ + 5 ZEATIN	100	4.53	100	5.47
38	TDZ-1	100	5.37	100	4.23
39	TDZ-5	100	6.83	96.67	6.67
	Std Error	3.29	0.84	1.688	0.57
	L.S.D	6.55	1.67	3.36	1.13

TABLE IV
SCORING FOR PERCENTAGE OF SOMATIC EMBRYOGENESIS AND NUMBER OF SOMATIC EMBRYO FORMED PER LEAF AND PETIOL EXPLANTS OVER THE DURATION OF EXPERIMENT (ONLY 4TH WEEK DATA ARE GIVEN AND TREATMENT NO. 10 TO 37 ARE NOT INCLUDED DUE TO THE ABSENCE OF SOMATIC EMBRYOGENESIS)

Treatment No.	Treatments (Concentration in μM)	WEEK 4 - Leaves		WEEK 4 - Petioles	
		Percentage Somatic Embryogenesis (%)	Number of Somatic Embryos formed	Percentage Somatic Embryogenesis (%)	Number of Somatic Embryos formed
1	Control	0	0	0	0
2	1 TDZ + 1 IAA	100	8.97	96.67	9.60
3	1 TDZ + 5 IAA	100	8.80	100	9.57
4	1 TDZ + 1 ZEATIN	96.67	5.77	90	7.53
5	1 TDZ + 5 ZEATIN	80	6.73	96.67	6.27
6	5 TDZ + 1 IAA	93.33	6.93	96.67	7.87
7	5 TDZ + 5 IAA	96.67	5.13	96.67	6.87
8	5 TDZ + 1 ZEATIN	96.67	5.30	93.33	6.97
9	5 TDZ + 5 ZEATIN	90	5.03	93.33	8.73
38	TDZ-1	96.67	6.60	100	6.27
39	TDZ-5	100	9.40	100	6.60
	Std Error	3.70	1.26	2.27	1.35
	L.S.D	7.36	2.51	4.51	2.69

Production of Plantlets

Somatic embryos derived from 10 TDZ treatments were retrieved to produce plantlets. The percentages of shoots and roots formed from somatic embryos were evidently higher in four treatments for both explants types (Table 5). Embryos originating from petioles produced noticeably higher number

of shoots than those derived from leaves. It was clear that treatments containing 1 μM TDZ in combination with 1 or 5 μM of IAA have produced the most efficient system for SE in *S. aemula* (Table 5). Prior to the transfer of the plantlets to soil, 36 plantlets were isolated and placed in jars regardless of the treatment orders. The survival rate of plantlets was at 100% (Fig. 4).

TABLE V
THE TOP FOUR TREATMENTS FOR INDUCTION OF SOMATIC EMBRYOGENESIS FROM PETIOLAR EXPLANTS OF *SCAEVOLA AEMULA*

Explants	Petioles			
Attributes	1 TDZ	5 TDZ	1 TDZ + 1 IAA	1 TDX + 5 IAA
Percentage of roots formed (No. of roots)	22 (0.53)	11.5 (0.8)	25.4(1.37)	48.7 (2.37)
Percentage of shoots formed (No. of shoots)	64.7 (2.2)	77.7 (2.67)	92.3(3.9)	98 (4.43)
Percentage of Somatic Embryogenesis	100	100	96.67	100
No. of somatic embryos formed	6.27	6.6	9.6	9.57

Fig. 4. Plantlets regenerated from somatic embryos of *Scaevola aemula* c.v. 'Purple Fanfare'.submission.

(b) Other factors influencing DSE : Effects of inorganic nutrient levels, media, pH and sugar

Callus Formation

Callus formation was the same for all treatments (100%) at week 5. However, in week 6 most of the treatments that have full MS as the media's basal salt plus high sucrose level (>30 g-1 L) have all their percentage dropped (<70%). The explants from these plates were senescing and their tissues blackening. The amount of callus was higher when the basal salt, pH and the sugar levels were increased. The highest amount of callus came from the treatment where all three properties (sugar, nutrients and pH) were at the highest level. The results demonstrated that the treatments containing half MS as basal salt callused more readily than full MS treatments.

Performance in Organogenesis

Organogenesis did not occur until the third week. Full MS seemed to have a detrimental effect on organogenesis. There were no significant differences between treatments ($P > 0.05$) of the pH levels and sucrose levels.

Production of Somatic Embryos

Two treatments chosen for this experiment (1 TDZ + 1 IAA and 1 TDZ + 5 IAA) produced somatic embryos. Starting from 3rd week, SE increased over the duration of the experiment. This trend was true for all treatments but the differences were in the number of somatic embryos formed and the percentage of explants with SE in particular treatments. Treatments utilizing full MS had lower numbers of somatic embryos and a lower percentage of explants with SE. Higher sucrose level did not inhibit SE but in fact improved the number produced. However embryogenesis was lower in treatments with higher levels of MS salts. Furthermore, at the end of the scoring period, blackening started to appear in the callus in full MS salts.

(c) Indirect Somatic Embryogenesis in Scaevola aemula

Callus formation was noticed in the first week after the explants had been placed on 2,4-D plates. The calli formed were very similar to the ones in the first experiment (a). Sugary white calli were formed in both explants types (Fig. 5). The amount of callus in all treatments for leaves was similar (Table 6). The same trend was noticeable for petioles (Table 6). The amount and percentage of callus formed were not different for the two explants types. In relation to organogenesis, the lower concentrations of 2,4-D produced higher percentage and number of organs in leaf explants (Table 6). However, in petioles higher concentrations of 2,4-D

induced organogenesis in more explants (%) than lower concentrations. Petioles produced more organogenesis than leaves in all of the treatments. For somatic embryogenesis in leaves, only the treatment with the use of charcoal (37A; Table 2) had embryos formed, however the percentage of explants (< 30%) and number formed were inefficient (Table 6). In petioles, two treatments with low concentration of 2,4-D produced somatic embryos (37 A and M) but still the efficiency was low (< 40%). There were no significant difference between the percentage and number of somatic embryos produced but it appears that petioles were more reactive to SE than leaves.

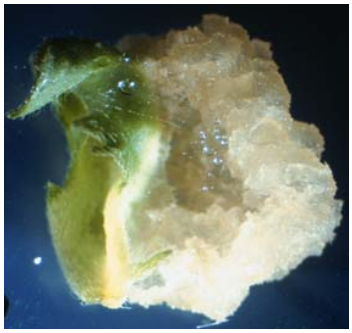

Fig. 5. Formation of callus from the treatment of 2, 4-D.

TABLE VI
SUMMARIZED DATA OF INDIRECT SOMATIC EMBRYOGENESIS FOR LEAF AND PETIOLE EXPLANTS IN *SCAEVOLA AEMULA*

Leaf explants

Treatments (A: Activated Charcoal; M: No Activated Charcoal)	Percentage Callus formed (%)	Amount of Callus (0-5)	Percentage Organogenesis (%)	Number of Organogenic structure formed / explants	Percentage Somatic Embryogenesis (%)	Number of Somatic Embryos formed / explants
37A	66.70	1	40.00	3.72	26.70	1.17
37M	66.70	1	53.30	3.83	0	0
38A	93.30	1	26.70	1.17	0	0
38M	100	1	26.70	1.67	0	0
Std Errors	16.33	0	24.49	1.313	9.43	0.51
L.S.D.	37.66	0	56.49	3.028	21.74	1.16

Petiole explants

Treatments (A: Activated Charcoal; M: No Activated Charcoal)	Percentage Callus formed (%)	Amount of Callus (0-5)	Percentage Organogenesis (%)	Number of Organogenic structure formed / explants	Percentage Somatic Embryogenesis (%)	Number of Somatic Embryos formed / explants
37A	60.00	1	60.00	5.56	13.30	0.33
37M	66.70	1.22	60.00	5.11	33.30	1.56
38A	86.70	1	86.70	4.27	0	0
38M	93.30	1	86.70	5.36	0	0
Std Errors	11.55	0.16	13.33	1.04	15.63	0.66
L.S.D.	26.63	0.36	30.75	2.40	36.05	1.52

IV. DISCUSSION

It is evident that TDZ can be used effectively to induce SE for *S. aemula*. Both 2, 4-D and IAA failed to induce SE in this experiment. However, it was possible to induce SE from leaf explants of *S. aemula* with 2, 4-D and BAP [8], [9]. With IAA treatments, the occurrence of direct regeneration was only noticeable when BAP was used in combinations with IAA. Similarly, BAP has been found to be useful in promoting the formation of multiple shoots of *S. aemula* R. Br. [6], [8]. Therefore, it is most likely that direct regeneration from explants was due to the effect of BAP. The present experiment showed that higher the BAP concentration, greater the direct regeneration. This factor has been seen in another species (*Dendranthema grandiflorum* (Ramat.) Kitamura) where the

number of adventitious shoots formed increased as the concentration of BAP increased [21].

With TDZ treatments, SE was induced in all treatments, but the efficiency of SE was concentration dependent. At 5 μM TDZ production of somatic embryos were less than in treatments with lower TDZ concentration (1 μM). Similarly, a lower concentration of TDZ was found to be more efficient for SE in *Havea brasiliensis* [22]. The efficiency of SE was improved when IAA was added to the treatments with TDZ. Other research indicated that TDZ concentration higher than 1 μM was supra optimal for Geranium [23], but in this study 5 μM of TDZ was not lethal but the production of somatic embryos were lower. A concentration of 1 μM of TDZ seemed to be the optimal concentration for somatic embryogenesis in *S. aemula*.

The production of shoots and roots became more efficient when IAA was added as supplemental auxin. However, production of shoots and roots appears to be less when somatic embryos are exposed to higher TDZ concentration treatments with *S. aemula*, hence there is possible inhibition, where TDZ concentration is supra optimal. In this study, TDZ has been shown to be the most effective PGR for inducing somatic embryogenesis in *S. aemula*. Petiole explants appear to be the best explants for SE with Purple Fan Flower.

The present results suggest that callus formation is optimal when half MS strength basal salts were used. Various pH and sucrose treatments showed no significant differences between treatments for callus production. However, at higher sucrose and pH the percentage of explants callusing increased. Similar results were reported in *Medicago arborea* L [24] where sucrose concentration affected callusing performance. In this study, results indicated that formation of callus requires a certain minimal basal salt and sucrose concentration since the cells require an exogenous source of energy for differentiation.

There were no significant differences between treatments in the initial phase of the experiment, but after week 4, evidence of drop in percentage of organogenesis started to appear in certain treatments with MS basal salts. There are two possibilities for this phenomenon. Firstly, the production of organogenesis is not only inhibited by the high levels of basal salts but may lead to death of tissues. Coincident with the former was a reduction in percentage of organogenesis (< 30%) from the lower pH treatments (5.5). Secondly, the production of somatic embryos was exceptionally vigorous in these treatments leading to a possible over-estimate of organogenesis that may have coincided with a pre-bipolar structure phase. It has been documented that ultrastructural changes during SE in pearl millet can produce differentiation and hence the initial structures can be mistaken for on set of organogenesis [25]. The production of somatic embryos was similar to organogenesis in that it occurred at week 3. The results again supported the fact that TDZ is an efficient inducer of SE in *S. aemula*, when the percentages of somatic embryos quickly increased to above 50% after week 4 for all treatments. The use of TDZ in producing somatic embryos in *Paulowina elongata* was reported up to 51% [26], which is similar to the observations in the present study with *S. aemula*. A significant increase in week 4 is an indication of the possibility of increased ultrastructural changes during organogenesis leading to SE. The results have provided some insight of *S. aemula* in response to SE.

The present study provided evidence that ISE can also occur in *S. aemula.* Production of somatic embryos was possible using callus derived from the explants. In another syudy it was reported that ISE goes through a callus phase before proceeding to the dedifferentiation phase [27]. The use of calli derived from immature embryos of soybeans as the initial material for SE indicated that ISE in *S. aemula* does not require incubation with activated charcoal as an intermediated step before the induction step providing that petioles are used as explants [28]. Activated charcoal was included to "remove" any remaining 2,4-D from the cells. The embryo production efficiency was lower than that from DSE. The fact that the higher the concentration of 2, 4-D inhibits SE indicated that the optimal level of 2,4-D was below 5 μM. Hence a wider range of concentrations needs to be evaluated in the future.

The duration taken for this species to react to ISE was comparatively short indicating that the production of ISE was efficient in this aspect. In a previous study, using callus phase to induce indirect somatic embryos in *Alstroemeria* spp.. it took at least 10 weeks for the callus induction to occur [29], [30]. In the present experiment callusing occurred in two weeks and the induction of embryogenesis a week later. This information indicates that the responsiveness of *S. aemula* in tissue culture is extremely good. This is only a preliminary experiment conducted to investigate the feasibility of ISE in *S. aemula*. There is future prospect of using callus for liquid culture and development of SE in liquid culture, which is considered the most efficient system for somatic embryo production.

In this research, a protocol was developed and the results indicated that production of direct somatic embryos as well as indirect somatic embryos in *S. aemula* could be achieved. The efficiency in the production of somatic embryos was improved by studying the factors (Basal salts strength, pH and sucrose levels) in the media used in the experiment. Although a previous study was successful in inducing SE from leaf explants of *S. aemula* [8], [9], the present study designated a protocol to obtain a high frequency of somatic embryos directly from petiole and leaf explants using an inductive PGR signal TDZ. Indirect somatic embryogenesis was also achieved. Thus, by following the protocol developed in the present study, the production efficiency of *S. aemula* could be improved in a large scale propagation system, in future.

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