RESEARCH ARTICLE

Efficient organogenesis from the induced meristemoid of *Anthurium andraeanum* Linden cv. Tinora

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Abstract
We present here an efficient micropropagation protocol through direct regeneration of plants from meristemoids in *Anthurium andraeanum* Linden cv. Tinora. About 96.6±0.33 of *in vitro* grown nodal segments having axillary buds were induced to form meristemoids on modified MS basal medium supplemented with 0.92 μM Thidiazuron (TDZ). The significantly highest numbers of shoots (25.6±0.23) were regenerated from 93.3±0.33% of meristemoids in the same culture medium. The histological and scanning electron microscopic (SEM) study confirmed direct organogenesis from the meristemoid.

Keywords: *Anthurium*; organogenesis; meristemoid; TDZ; SEM; histology

Introduction

The herbaceous, evergreen and perennial *Anthurium andraeanum* Linden cv. Tinora is very lucrative for its long lasting, attractive, striking vibrant inflorescence with straight spathe, candle-like spadix and exotic foliage (Chen *et al.,* 2011). Conventional propagations cannot be able to fulfil the high market demand of this plant. Therefore,

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micropropagation is the only option for large scale commercial production.

In vitro regeneration of any plant has been obtained via two major pathways *i.e.* organogenesis and somatic embryogenesis (Duclercq *et al.*, 2011). Typically organogenesis is evident either by the appearance of small precursor cell populations termed as meristemoids (Thorpe, 1993) and gradually, these meristemoids transform into shoot, root, or floral apical meristems. Thus, meristemoids can act as plant stem cells and they have transient self-renewing capacity (Pillitteri *et al.,* 2011). Many studies have been documented for efficient direct and indirect organogenesis and regeneration system of *A. andraeanum* (Beyramizade *et al.,* 2008; Gu *et al.,* 2012). The appearance of meristemoid like structure during direct shoot bud regeneration from young brown lamina (Martin *et al.,* 2003) and callus mediated regeneration (Joseph *et al.,* 2003) of *A. andraeanum* were reported earlier. However the detailed studies of meristemoid formation, multiplication and shoot and root regeneration from them were not documented. Furthermore, there is no report on histological and SEM analysis of meristemoids and shoot bud differentiation from meristemoid.

Here, we report on the optimization of parameters for the initiation, multiplication of meristemoid and subsequent regeneration of plants from nodal segment of *in vitro* established *A. andraeanum* plants. The anatomical and structural features of meristemoid and the process of organogenesis have been substantiated with the help of histology and SEM study.

Materials and methods

The nodal segments having axillary bud (1cm), from previously established 10-12 months old *in vitro*-grown plantlets of *A. andraeanum*, were dissected and vertically placed on culture medium for induction of meristemoid (Fig.1a). The basal medium used in all the experiments consisted of half strength of macro elements and full strength of other Murashige and Skoog's (1962) inorganic salts and vitamins, 3% (w/v) sucrose, 0.8% (w/v) agar (Bacteriological grade, Himedia) and has been defined as

MMS (Modified MS) in the present paper. The media pH was adjusted to 5.8. The cultures were incubated under cool, white fluorescent lights (16 h photoperiod; 55 µmol m^{-2} s^{-1}, Philips, India) at 25±2.0°C and 70% relative humidity (RH).

The MMS media along with a range of N$_6$-Benzyl adenine (BA) (0.44-2.21 µM), Indole acetic acid (IAA) (0.57-1.71 µM), Kinetin (KN) (0.46-1.84 µM), α-Naphthalene acetic acid (NAA) (0.27-2.16 µM) and TDZ (0.46-1.32 µM), either alone or in combination, were used for meristemoid induction, multiplication and subsequent regeneration of plantlets. Initially, the explants were cultured for 8 weeks to induce meristemoid. The induced meristemoids were cut into two pieces and subcultured at every 4 weeks on TDZ (0.46 µM) supplemented MMS medium for multiplication. The multiplied meristemoids were further cultured in different plant growth regulator (PGR) containing fresh media (Table 1) for another 8 weeks to regenerate shoots and roots.

The regenerated plantlets measuring a size of about 6-8 cm having 8-10 fully expanded leaves and 2-3 aerial roots were directly harvested from the culture and transferred to the portrays with a mixture of coco peat, sand and vermiculite in the ratio of 1:1:1 (v/v/v) for primary hardening in the controlled greenhouse and kept for 8 weeks. The individual hardened plant with intact root ball transferred to a plastic bag (15 x 10 cm) containing chopped coco husk and broken pieces of charcoal and kept under 75% agro shade net for another 6 weeks before final shifting in the field condition.

A standard procedure as described by Paul *et al.,* 2011 was followed for histological and SEM study of 8 weeks old meristemoids from initial culture medium and 20 weeks old meristemoids from regeneration medium.

Ten numbers of nodal segments were used for induction of meristemoids in each trial. The ability of meristemoid induction was recorded after 8-weeks of culture. The shoot bud differentiation and average numbers of shoots with aerial roots were counted from the meristemoid finally after second passage *i.e.* at the end of 8 weeks of culture in fresh medium. All sets of experiment were repeated thrice. All experimental data were subjected to analysis of variance (ANOVA) and significant (P < 0.05) means were determined with Duncan's multiple range test (DMRT) to distinguish differences between treatment means at the α = 0.05 level using Statistical Package for the Social Sciences (SPSS) for windows, version 16.

Results and Discussion

Initially meristemoid was originated as a hard, compact bulbous structure at the basal portion of the nodal segments (Fig. 1a) within 2 weeks of inoculation. MMS media supplemented with BA (0.88-2.21 µM), NAA (0.53-2.16 µM), KN (0.92-1.84 µM), IAA (0.57-1.71 µM), TDZ (0.46-1.32 µM) either alone or in combination induced meristemoid from 3.3±0.33 to 96.6±0.33% of explants

Table 1. Role of Modified MS supplemented PGRs on meristemoid induction and regeneration of plantlets of *Anthurium andraeanum* Linden cv. Tinora.

PGR Concentration (µM)					% of responded explants[a]	% of regenerated meristemoid[b]	Average No. of plants / meristemoid[c]
BA	NAA	IAA	KN	TDZ			
0.00	0.00	0.00	0.00	0.00	0.0h	0.0f	0.0e
0.44	0.27				0.0h	0.0f	0.0e
0.88	0.53				23.3±0.33def	66.0 ±0.66c	4.3 ±0.13de
1.33	1.0				10.0 ±0.57gh	33.3±0.33de	0.0e
1.77	1.62				13.3±0.33fgh	23.3±0.33 e	10.0±0.1bc
2.21	2.16				33.3±0.33cd	33.0 ±0.33de	7.3±0.2cd
			0.46		0.0h	0.0f	0.0e
			0.92		33.3±1.3cd	0.0f	0.0e
			1.38		43.3±0.66bc	0.0f	0.0e
			1.84		40.0±0.1bcd	0.0f	0.0e
0.88		0.57			36.6±0.33cd	0.0f	0.0e
1.33		1.14			3.3±0.33h	0.0f	0.0e
1.77		1.71			30.0±0.0cde	0.0f	0.0e
2.21		1.71		1.38	23.0±0.33def	0.0f	0.0e
				0.46	56.6±0.33b	83.3 ±0.33 ab	15.3±0.17b
				0.92	96.6±0.33a	93.3±0.33a	25.6±0.23a
				1.32	43.3±0.66bc	76.6±0.33bc	0.0e

[a] Data was scored after 8-weeks of culture in the initial experiment. [b & c] Data was recorded after 8-weeks of culture from the second passage. Values are mean ± standard error of three replicated experiments. Means followed by the same letter are not significantly different at P < 0.05 according to the Duncan multiple range test.

(Table 1). The morphological feature and size of the meristemoids varied in different culture media. However, TDZ (0.92 µM) supplementation, in comparison to other PGRs, showed significantly best response where 96.6±0.33% of explants induced meristemoids. The induced meristemoid were also successfully multiplied for another 4 weeks in fresh MMS medium supplemented with 0.46 µM of TDZ (data has not shown). In the multiplication medium the meristemoids were enlarged and looked harder, compact, and slightly irregular in shape with occasional shoot bud differentiation (Fig.1b). In a set of fresh culture media, the meristemoids induced shoot buds (Fig. 1c) more or less within 2 weeks of culture. The culture media augmented with TDZ (0.46- 0.92 µM) or BA (0.88-2.21 µM) plus NAA (0.53-2.16 µM) induced shoots at varying frequencies (Table 1) where, the other combinations of PGRs (BA+IAA, BA+IAA+KN, and KN alone) did not respond at all. Significantly the highest numbers of shoots (25.6±0.23) were regenerated from 93.3±0.33% of meristemoids in TDZ (0.92 µM) containing culture medium. The regenerated shoot buds and microshoots enclosed the entire surface of meristemoid within 6 weeks of culture (Fig. 1d) and in the next two weeks, large numbers of microplants along with aerial roots appeared on them (Fig. 1e). The plantlets reached a size of about 6-8 cm having 8-10 fully expanded leaves and 2-3 aerial roots (Fig. 1f) were directly harvested from the culture and hardened within green house with the described protocol.

Many reports indicate the formation of meristemoid on callus or directly on explant. During *in vitro* organogenesis from leaf explants of *A. andraeanum,* Joseph *et al.* (2003) reported the appearances of green spotted meristemoids on the callus after 50 days of subculture in half strength MS medium either alone or supplemented with combinations of BA, 2, dichlorophenoxyacetic acid (2,4-D) and KN. Martin *et al.* (2003) also detected 3-6 meristemoids of *A. andraeanum* at the time of direct shoot regeneration from lamina explants by using half strength MS medium augmented with BA, IAA, and kinetin.

The direct shoot regeneration in *A. andraeanum* from micro-cuttings was obtained by using BA with NAA (Vargas and Mejías, 2004; Raad *et al.,* 2012). In the present study, TDZ (0.92 µM) successfully induced meristemoid from the cut end of the explant which was nothing but a suppressed meristem. From this tissue mass on an average 25-30 plants directly regenerated at 8 week subculture period. Commonly some definite cells in a primary explant may be converted directly into small precursor cell population called meristemoid (Hicks, 1994).

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In the present report, the histological images revealed that the competent dividing cells were located at the outer surface of the meristemoid which were smaller in size than the surrounding cells and contain densely stained nuclei (Fig. 1g). A good number of shoot bud asynchronously differentiated from the outer surface of the meristemoid (Fig. 1h). The mature shoot bud regeneration from meristemoid was also observed and they are deeply sited within it (Fig. 1i) which is a characteristic feature of organogenesis. The SEM studies of meristemoid also revealed tightly packed epidermal cells, formation of large number of meristematic nodules with definite plane of divisions (Fig. 1j), and shoot buds differentiation on the nodules (Fig. 1k). Similar histological feature of meristemoid formation was observed in Tobacco (Altamura *et al.,* 1995) where they demonstrated the large meristemoid formation in a superficial proliferative area. Confirmation of organogenesis by SEM analysis was also carried out in plant like *Rumex* sp. (Slesak´ *et al.,* 2014).

Conclusion

In this study, we established a simple, highly efficient, reproducible and cost effective protocol for direct organogenesis and subsequent regeneration of plants from *in vitro* nodal segments of *A. andraeanum* through the formation of meristemoids. Organogenesis and subsequent shoot regeneration were further confirmed by histological and SEM analysis.

Competing interests

The authors declare that they have no competing interests.
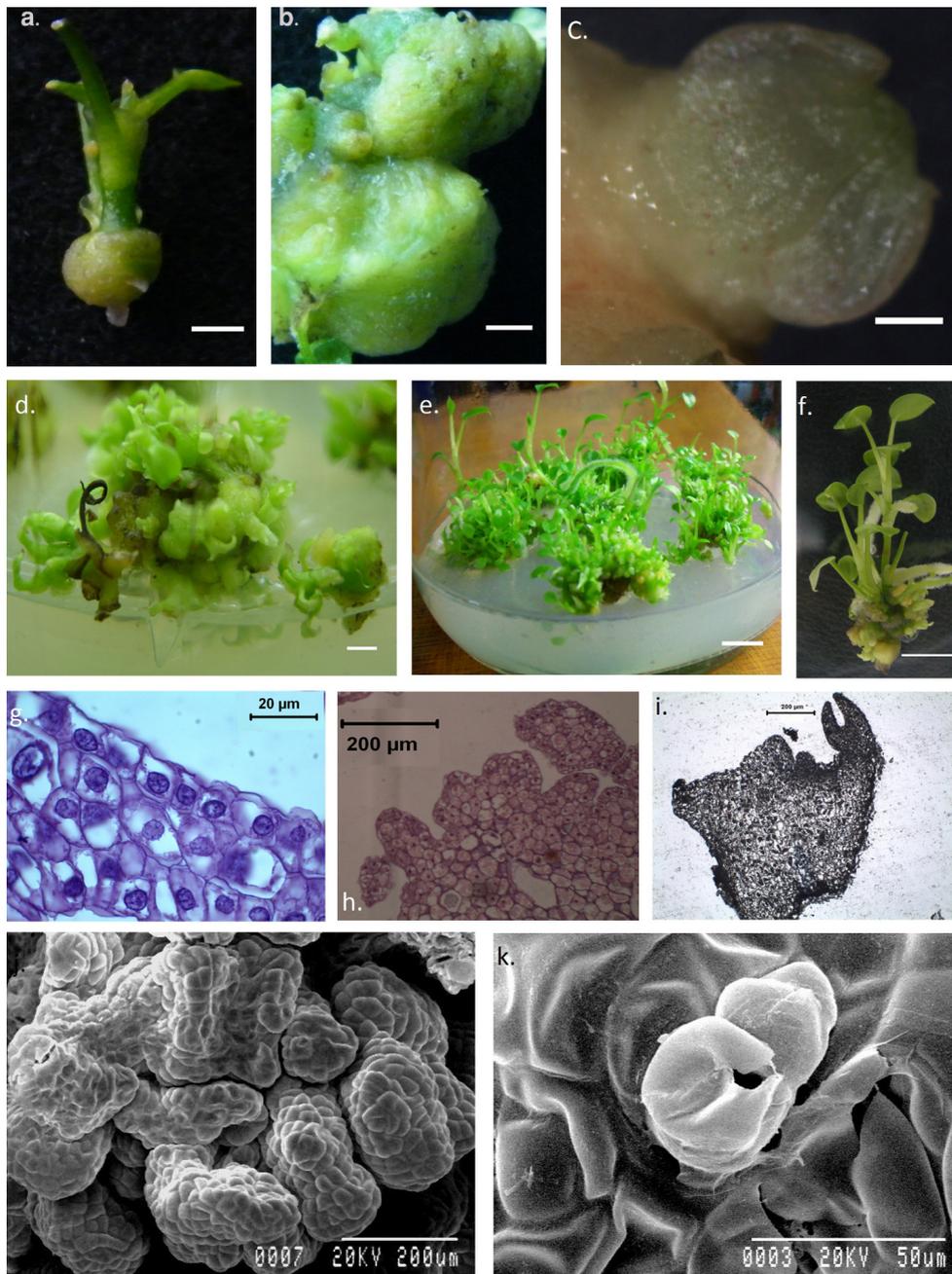
Fig. 1. Differentiation of meristemoid from *in vitro* grown nodes and subsequent regeneration of plantlets in *Anthurium andraeanum* Linden cv. Tinora. a. induction of bulbous meristemoid at the cut end of the nodal segments, in Modified MS medium supplemented with TDZ (0.92 µM) Scale Bar = 1 cm; **b.** enlarged, hard and compact meristemoid after culture in multiplication medium having MMS with 0.46 µM TDZ, Scale Bar = 1 cm; **c.** enlarged view of shoot bud differentiation from meristemoid, Scale Bar = 1 cm; **d.** regenerated shoot buds and microshoots covered the entire surface of meristemoid after culture in MMS medium with TDZ (0.92 µM) Scale Bar = 1 cm; **e.** development of large number of shoots along with aerial roots on the meristemoids **f.** well-developed plants with 2-3 aerial roots, ready for harvesting, Scale Bar = 2 cm; **g. – i.** the histological images; **g.** competent dividing cells containing densely stained nuclei located at the outer surface of the meristemoid; **h.** asynchronous differentiation of shoot buds from the meristemoid; **i.** shoot bud regenerated from meristemoid are deeply sited within it, **j. – k.** the SEM images **j.** tightly packed cells of outer surface and large number of meristematic nodules with definite plane of divisions; **k.** shoot buds differentiated from the meristematic nodule.

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