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Original Article

Studies on the effect of individual plant growth regulators on *in vitro* culture of *Taraxacum officinale*

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Abstract

Taraxacum officinale is well known for its medicinal properties. Using different combinations of auxins and cytokinins, callus induction and regeneration has been reported previously. However, studies on the effect of individual plant growth regulators are limited. In the present study, individual effect of auxins (2,4-D and NAA) and cytokinins (kinetin, BAP and TDZ) on callus induction and plant regeneration was tested. Leaf and root explants cultured on MS medium plus 0.5 mg/L 2,4-D produced greenish white and light brown friable callus and those cultured on NAA induced pigmented callus. This is the first report on the induction of pigmented callus in *T. officinale* using NAA alone. Kinetin caused direct shoot regeneration whereas BAP and TDZ induced direct as well as indirect shoot regeneration. Regenerated plantlets were acclimatized successfully at 20 ± 2 °C. Scanning electron microscopy (SEM) revealed differences in stomata and leaf surface of *in vitro* and acclimatized plantlets.

Keywords: *Taraxacum officinale*, plant regeneration, callus, auxin, cytokinin, acclimatization

1. Introduction

Taraxacum officinale (L.) Weber ex F.H. Wigg. (Asteraceae), known as common dandelion, is a cosmopolitan herb distributed worldwide, especially in the temperate zones of the northern hemisphere with warm climate. It has been used in traditional medicine for its health-promoting properties such as choleretic, diuretic, anti-rheumatic, anti-inflammatory, anti-carcinogenic, laxative and hypoglycemic activities (Schütz, Carle, & Schieber, 2006). Various parts of the plant are edible and are used in salads (Escudero, De Arellano, Fernández, Albarracín, & Mucciarelli, 2003) and as a substitute for coffee (Sweeney, Vora, Ulbricht, & Basch, 2005). Moreover, a closely related species, *Taraxacum kok-saghyz* produces high molecular weight rubber with properties comparable to *Hevea brasiliensis* (Gronover, Wahler, & Prüfer, 2011). Hence, *T. officinale* can be used as a suitable model plant for the functional genomics studies of rubber biosynthesis.

The diverse importance of *T. officinale* demands the establishment of a proper *in vitro* culture system for this plant. Direct and indirect plant regeneration (Bowes, 1970; Chen, Li, Liu, & Li, 2005; Ermayanti & Martin, 2011; Gou, Kim, & Hong, 2009) as well as secondary metabolite production from callus (Akashi, Saito, Hirota, & Ayabe, 1997) and *in vitro* plants of *T. officinale* (Jamshieed, Das, Sharma, & Srivastava, 2010) has been reported previously. However, in most of the previous studies, the regeneration or accumulation of secondary metabolites was induced by combinations of plant growth regulators. Studies on the effect of individual plant growth regulator may provide a better understanding of the regulation of various metabolic pathways leading to morphogenesis as well as the production of bioactive compounds, which could be utilized in further metabolic engineering programs. Towards this goal, individual effect of five different

plant growth regulators on callus induction and plant regeneration of *T. officinale* was evaluated in the present study.

2. Materials and Methods

2.1 Establishment of Aseptic Explants

Seeds of *T. officinale* were collected from Lund region of Sweden. The seeds were soaked in distilled water for five minutes followed by surface sterilization with 10% Clorox™ (v/v) (NaOCl) containing 2-3 drops of Tween-20 for five minutes. The seeds were then rinsed in sterile distilled water three times and were inoculated onto basic Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) without any plant growth regulator. The *in vitro* plantlets obtained from the seeds were subcultured to fresh MS medium every four weeks.

2.2 Determination of the Effect of Individual Plant Growth Regulators (PGRs)

Leaf (1 cm x 1 cm) and root explants (1 cm) of three-week-old *T. officinale* seedling were inoculated onto MS medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D), 1-naphthaleneacetic acid (NAA), kinetin, 6-benzyl aminopurine (BAP) and thidiazuron (TDZ) individually at different concentrations (0.5, 1.0, 1.5, 2.0 mg/L). The pH of the medium was adjusted to 5.7-5.8 using 1M HCI or NaOH prior to autoclaving at 121ºC for 15 minutes. All the PGRs were added prior to autoclaving except for TDZ which was filter-sterilized and added to the medium after autoclaving. Cultures were incubated in a culture room at 27 ± 2 ºC and under 16 hour photoperiod with a light intensity of 3.08 Klux provided by cool white fluorescent tubes. There were seven replicates for each concentration tested and each replicate contained

three explants. The explants cultured on MS medium devoid of plant growth regulators served as control. The fresh weight of the explants with callus, roots and shoots as well as the number of regenerated shoots per explant were recorded after four weeks of culture. Two-way analysis of variance (ANOVA) was used to determine the significant difference between the treatments. Tukey's test was used to compare the differences among the means using IBM SPSS statistic 22.0.

2.3 Acclimatization

Regenerated shoots without roots were transferred to MS basal medium for rooting. Regenerated plantlets with 2-3 roots were removed from the culture vessels and washed with tap water to remove the agar sticking on the surface of the roots. The plantlets were transferred to the pots containing garden soil and covered with plastic bottles to maintain the humidity. The plantlets were watered twice a day using tap water. The acclimatization was carried out under two conditions, with one set of the plantlets acclimatized in the growth chamber with temperature 20 ± 2 ºC and relative humidity 44% while the other set of plantlets was acclimatized under room temperature (30 ± 2 ºC) and relative humidity 39%. Ten plants were used for each acclimatization condition. The percentage of survival of the acclimatized plantlets was recorded after four weeks.

2.4 Observation of Leaf Morphology by Scanning Electron Microscope (SEM)

For scanning electron microscopy (SEM), leaves of *T. officinale* (*in vitro*, growth chamber acclimatized and room temperature acclimatized) were freeze dried. The observation of leaf morphology was carried out using a Leo Supra 50 VP field emission scanning electron microscope (CarlZeiss SMT, Oberkochen, Germany) operated at 12 kV, with a working distance of 15 mm.

3. Results & Discussion

3.1 Determination of the Effect of Individual Plant Growth Regulators (PGRs)

Table 1 shows the effect of auxins, 2,4-D and NAA, on culture response of the leaf and root explants of *T. officinale*. Leaf and root explants cultured on different concentrations of 2,4-D showed callus formation at the cut ends of the explants. Fresh weight of callus obtained from leaf explants cultured on MS medium fortified with 0.5, 1.0 and 1.5 mg/L 2,4-D were significantly higher than that of 2.0 mg/L 2,4-D. However, there was no significant difference among those three concentrations of 2,4-D after four weeks of culture. Fresh weight of callus obtained from the leaf and root explants ranged from 0.07 – 0.17 g and 0.04 – 0.06 g, respectively. The percentage of callus induction from leaf and root explants were 76-86% and 95-100%, respectively. Leaf explants produced greenish white friable calli and root explants produced light brown friable calli. 2,4-D is commonly used for callus induction of grasses and herbs (Bhaskaran & Smith, 1990). In the present study, 0.5 mg/L 2,4-D was the best concentration for producing the highest callus fresh weight from both leaf and root explants. Similarly, low concentration of 2,4-D was able to induce callus from leaf explants of *Carthamus tinctorius* (Kumari, Pandey, & Uttam, 2015).

Leaf explants cultured on the MS medium supplemented with different concentrations of NAA produced whitish green friable calli with random spots of red pigmentation. When the concentration of NAA was increased to 1.5 and 2 mg/L, root formation was observed. In the case of root explants, purplish red friable calli was observed (Figure 1A). There was sporadic shoot formation from root explants cultured in different concentrations of NAA containing medium. In terms of callus fresh weight

derived from leaf and root explants, there was no significant difference among different concentrations of NAA. The fresh weight of callus from the leaf and root explants cultured on different concentrations of NAA ranged from 0.51 – 0.99 g and 0.18 – 0.60 g, respectively. The percentage of callus induction from leaf and root explants were 86-95% and 100%, respectively. NAA is a commonly used auxin for rooting of *in vitro* shoots (Ďurkovič & Bukovská, 2009). Leaf and root explants cultured on all the concentrations of NAA showed callus formation with varying degree of callus growth. Callus induction on MS medium supplemented with NAA has been previously reported for *Withania somnifera* (Adhikari & Pant, 2013). In the present study, purplish red callus was induced from the leaf and root explants cultured on different concentrations of NAA. The induction of purplish red callus in *T. officinale* under the influence of NAA alone has not been reported before. However, pigment induction on cytokinin-rich medium has been reported (Akashi *et al*., 1997). On the other hand, it has been reported that NAA had the ability to stimulate anthocyanin production in callus of *Rudbeckia hirta* (Luczkiewcz & Cisowski, 2001).

Table 2 shows the effect of cytokinins on the leaf and root explants of *T. officinale.* Leaf and root explants cultured on MS medium supplemented with different concentrations of kinetin (0.5, 1.0, 1.5, 2.0 mg/L) showed direct shoot regeneration. An average of 2-3 fully developed shoots per explant was recorded after four weeks of culture in all the concentrations of kinetin (Figure 1B). Few roots were observed sporadically in the regenerated shoots and there was no callus formation on both explants. There was no significant difference among different concentrations of kinetin on the fresh weight of both explants with shoots after four weeks of culture. Average fresh weight of the leaf and root explants with shoots cultured on different concentrations of kinetin were in a

range of 0.70 – 1.48 g and 0.42 – 0.69 g, respectively. The percentage of shoot regeneration from leaf and root explants cultured on different concentrations of kinetin were 67-90% and 76-86%, respectively.

When the leaf and root explants were cultured on MS medium supplemented with different concentrations of BAP, no friable callus or root formation was observed. Instead, direct and indirect shoot regeneration were observed. Different stages of the somatic embryo development such as the globular, heart-shaped, torpedo-shaped and cotyledonary stages were observed in both explants cultured on BAP-supplemented medium (Figure 2). Direct regeneration of shoots by BAP was also reported in *Solidago canadensis* (Li, Kang, Qiang, & Peng, 2012). Similar to kinetin, root and leaf explants produced an average of 1-3 fully developed shoots on medium supplemented with different concentrations of BAP. Fresh weight of the leaf explants with shoots cultured on MS medium plus 1.5 mg/L BAP was significantly lower than that of 0.5 and 1.0 mg/L BAP. Fresh weight of the root explants with shoots cultured on MS medium plus 2.0 mg/L BAP was the lowest among all the tested concentrations of BAP. Average fresh weight of the leaf and root explants with shoots cultured on different concentrations of BAP were in a range of 0.94 – 1.87 g and 0.73 – 1.14 g, respectively after four weeks of culture. The percentage of shoot regeneration from the leaf explants was 82-100% while 80-95% was recorded for root explants.

Kinetin and BAP are cytokinins which promote shoot regeneration (Tiwari, Tiwari, & Singh, 2001). In the present study, best culture response among the kinetin treatments was observed on the leaf explants cultured on MS medium enriched with 2.0 mg/L kinetin, as it obtained the highest fresh weight with 90% of shoot regeneration. Similar observations were obtained in *Sphaeranthus indicus* (Yarra *et al.,* 2010). In the

case of BAP, leaf explants cultured on 0.5 mg/L gave better culture response than root explants in terms of fresh weight of explant with shoots, number of regenerated shoots and percentage of shoot regeneration. This result was in accordance to the study conducted in *Arnica montana* (Surmacz-Magdziak & Sugier, 2012).

When leaf and root explants were cultured on MS medium supplemented with different concentrations of TDZ, no root formation was observed. This observation was similar to BAP treatments. Direct shoot regeneration and induction of embryogenic calli from both explants were observed in TDZ-supplemented medium. No significant difference was found among different concentrations of TDZ (0.5, 1.0, 1.5 and 2.0 mg/L) in terms of fresh weight obtained from leaf and root explants. Average fresh weight of the explants with shoots cultured on different concentrations of TDZ ranged from 1.91 – 2.33 g and 1.28 – 1.73 g, respectively after four weeks of culture. An average of 1-3 fully developed shoots were obtained from both explants cultured on TDZ containing medium and the percentage of shoot regeneration from both explants were around 80-100%, respectively. TDZ helps to establish the optimal endogenous levels of cytokinin and auxin needed for the induction of somatic embryos (Saxena, Malik, & Gill, 1992). In the current study, the culture response of leaf explants inoculated on MS medium enriched with 0.5 mg/L TDZ was better than other tested TDZ concentrations in terms of the shoot regeneration. Similar observation was also found in *Digitalis lamarckii* (Verma, Yücesan, Cingöz, Gürel, & Gürel, 2011). In *Pluchea lanceolate*, nodal explants cultured on MS medium plus 0.5 mg/L TDZ showed the highest multiplication rate (Kher, Joshi, Nekkala, Nataraj, & Raykundaliya, 2014).

When compared to kinetin, leaf explants cultured on TDZ containing medium had better culture response in terms of all culture parameters and it was superior to BAP in

terms of fresh weight of the explants with shoots. Moreover, leaf was better explant than root for plantlet regeneration in terms of all culture parameters. Thus, TDZ was the best PGR for plantlet regeneration of *T. officinale* using leaf explants. As compared to the other types of cytokinins, TDZ is a non-purine compound that exhibits good cytokinin-like activity. It has been commonly used for shoot organogenesis and proliferation of wide variety of plant species (Khawar, Sancak, Uranbey, & Özcan, 2004).

3.2 Acclimatization

When the regenerated shoots in kinetin, BA and TDZ were transferred to MS medium without the addition of PGR, it produced elongated roots. *In vitro* shoots of *Elaeagnus angustifolia* were reported to be rooted in auxin-free MS medium (Iriondo, De La Iglesia, & Pérez, 1995). When the complete plantlets of *T. officinale* were acclimatized at 20 ± 2 °C, 100% survival rate was recorded (Figure 3) while the plants acclimatized at 30 ± 2 °C showed only 20% survival rate. *T. officinale* grows very well under lower temperature in its natural habitat in the temperate hemispheres. Hence, the survival rate at higher temperature would be low.

3.3 Observation of Leaf Morphology by Scanning Electron Microscope (SEM)

Figure 4 clearly shows that the leaf morphology of the *in vitro* and acclimatized plants were different in the aspect of stomata and leaf lamina. The surface of the leaves of *in vitro* plants was smooth (Figure 4A) whereas the leaf surface of acclimatized plants was more wrinkled in nature due to the deposition of epicuticular wax (Figure 4B & C). The relative humidity used for *in vitro* plantlets were about 50-60% whereas for the acclimatized plants were about 39-44%. The reduction in relative humidity might have caused the acclimatized plants to accumulate more epicuticular wax (Lamhamedi,

Chamberland, & Tremblay, 2003). The stomata of the *in vitro* leaves were bigger and oval-shaped surrounded by kidney-shaped guard cells (Figure 4D) whereas the stomata of the leaves of acclimatized plants were smaller and elongated with elliptical and sunken guard cells (Figure 4E & F). Similar observation was reported for rose (Capellades, Fontarnau, Carulla, & Debergh, 1990). In terms of the number of stomata, *in vitro* leaves apparently had higher number of stomata than those from acclimatized plants. Similar observations were also found in *Ficus carica* (Chirinéa, Pasqual, Araujo, Pereira, & Castro, 2012) and *Tabebuia roseo-alba* (Porto *et al.*, 2014). The *in vitro* culture is characterized by high humidity, low light intensity and abundant nutrients along with PGRs. The closed culture condition prevents the entry of microorganisms and also reduces air turbulence which indirectly increases the boundary layers of the leaf and restricts the inflow and outflow of gaseous products. Consequently, this specialized culture conditions resulted in the abnormal development of plantlets in the aspect of anatomy, morphology and physiology such as poor stomatal development, photosynthetic organelles as well as cuticle formation (Pospóšilová, Tichá, Kadleček, Haisel, & Plzáková, 1999).

4.0 Conclusions

Pigmented callus of *T. officinale* was initiated on MS medium supplemented with NAA. Regeneration of plantlets were successful when the leaf and root explants were cultured on MS medium supplemented with BA, kinetin and TDZ individually. Significant changes were observed in the stomata and leaf surface of the *in vitro* and two different methods of acclimatized plants.

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Table 1. Effect of auxins (2,4-D and NAA) on callus induction from leaf and root explants of *T. officinale*

Type of PGR	Concentration of PGR (mg/L)	Fresh weight of the explant with callus/roots (g)	Callus induction (%)
Leaf explant			
2,4-D	0	0.00 ± 0.00 b	0
	0.5	0.17 ± 0.04 a	76
	1.0	0.12 ± 0.03 a	76
	1.5	0.16 ± 0.04 a	86
	2.0	0.07 ± 0.01 b	76
NAA	0	0.00 ± 0.00 b	0
	0.5	0.54 ± 0.13 ab	90
	1.0	0.51 ± 0.19 ab	95
	1.5	0.97 ± 0.26 a	86
	2.0	0.99 ± 0.16 a	86
Root explant			
2,4-D	0	0.00 ± 0.00 b	0
	0.5	0.06 ± 0.06 a	100
	1.0	0.04 ± 0.01 a	95
	1.5	0.05 ± 0.01 a	100
	2.0	0.04 ± 0.01 a	95
NAA	0	0.00 ± 0.00 a	0
	0.5	0.60 ± 0.31 a	100
	1.0	0.20 ± 0.06 a	100
	1.5	0.18 ± 0.15 a	100
	2.0	0.33 ± 0.20 a	100

Data were mean ± SE of seven replicates. Values with different letters within each group are significantly different ($p<0.05$) based on Tukey's test.

Table 2. Effect of cytokinins (kinetin, BA and TDZ) on the shoot regeneration of leaf and root explants of *T. officinale*.

Type of PGR	Concentration of PGR (mg/L)	Fresh weight of the explant with callus/shoots/roots (g)	Mean number of shoots per explant	Shoot regeneration (%)
		Leaf explant		
Kinetin	0	0.00 ± 0.00 b	0 ± 0.00 b	0
	0.5	1.31 ± 0.60 a	2 ± 0.64 a	67
	1.0	0.80 ± 0.20 ab	2 ± 0.31 a	86
	1.5	0.70 ± 0.13 ab	2 ± 0.29 a	86
	2.0	1.48 ± 0.23 a	2 ± 0.18 a	90
BA	0	0.00 ± 0.00 c	0 ± 0.00 b	0
	0.5	1.70 ± 0.25 a	3 ± 0.30 a	100
	1.0	1.87 ± 0.19 a	3 ± 0.31 a	100
	1.5	0.94 ± 0.18 b	2 ± 0.08 a	82
	2.0	1.42 ± 0.24 ab	2 ± 0.44 a	95
TDZ	0	0.00 ± 0.00 b	0 ± 0.00 b	0
	0.5	2.33 ± 0.22 a	3 ± 0.20 a	100
	1.0	1.91 ± 0.19 a	3 ± 0.37 a	100
	1.5	2.14 ± 0.21 a	3 ± 0.37 a	82
	2.0	2.31 ± 0.20 a	2 ± 0.26 a	95
		Root explant		
Kinetin	0	0.00 ± 0.00 b	0 ± 0.00 b	0
	0.5	0.42 ± 0.13 ab	2 ± 0.29 a	86
	1.0	0.65 ± 0.16 ab	3 ± 0.60 a	81
	1.5	0.69 ± 0.25 a	3 ± 0.61 a	86
	2.0	0.60 ± 0.22 ab	2 ± 0.67 a	76
BA	0	0.00 ± 0.00 b	0 ± 0.00 c	0
	0.5	1.14 ± 0.20 a	2 ± 0.26 a	95
	1.0	0.97 ± 0.24 a	2 ± 0.30 ab	90
	1.5	0.92 ± 0.20 a	1 ± 0.18 ab	80
	2.0	0.73 ± 0.22 b	1 ± 0.26 b	81
TDZ	0	0.00 ± 0.00 b	0 ± 0.00 c	0
	0.5	1.61 ± 0.19 a	2 ± 0.29 ab	100
	1.0	1.73 ± 0.14 a	2 ± 0.14 a	100
	1.5	1.40 ± 0.19 a	2 ± 0.18 ab	100
	2.0	1.28 ± 0.23 a	1 ± 0.26 b	81

Data were mean ± SE of seven replicates. Values with different letters within each group are significantly different ($p<0.05$) based on Tukey's test.

FIGURE LISTS

Figure 1 Effect of NAA and kinetin on *in vitro* response of *T. officinale*

Figure 2 Different stages of somatic embryo development observed on leaf and root explants of *T. officinale* cultured on MS medium supplemented with BAP

Figure 3 Acclimatized *in vitro* plantlets of *T. officinale* in the growth chamber after four weeks.

Figure 4 View of abaxial leaf surface and stomata of *T. officinale*

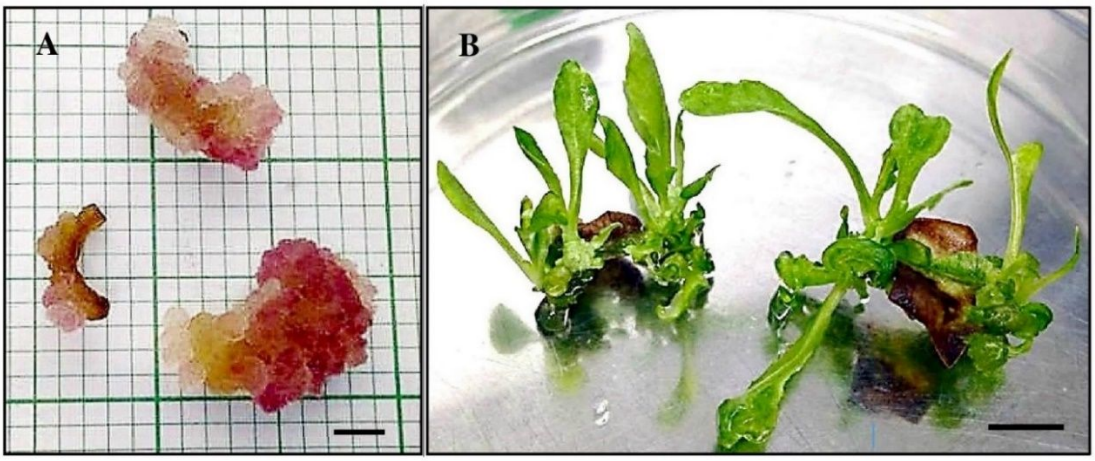

Figure 1. Effect of NAA and kinetin on *in vitro* response of *T. officinale*. (A) Purplish red pigmented callus initiated from root explants cultured on MS medium supplemented with 1.5 mg/L NAA. Scale bar= 4 mm, (B) Shoots regenerated from leaf explants of *T. officinale* cultured on MS medium supplemented with 2.0 mg/L kinetin. Scale bar= 1 cm.

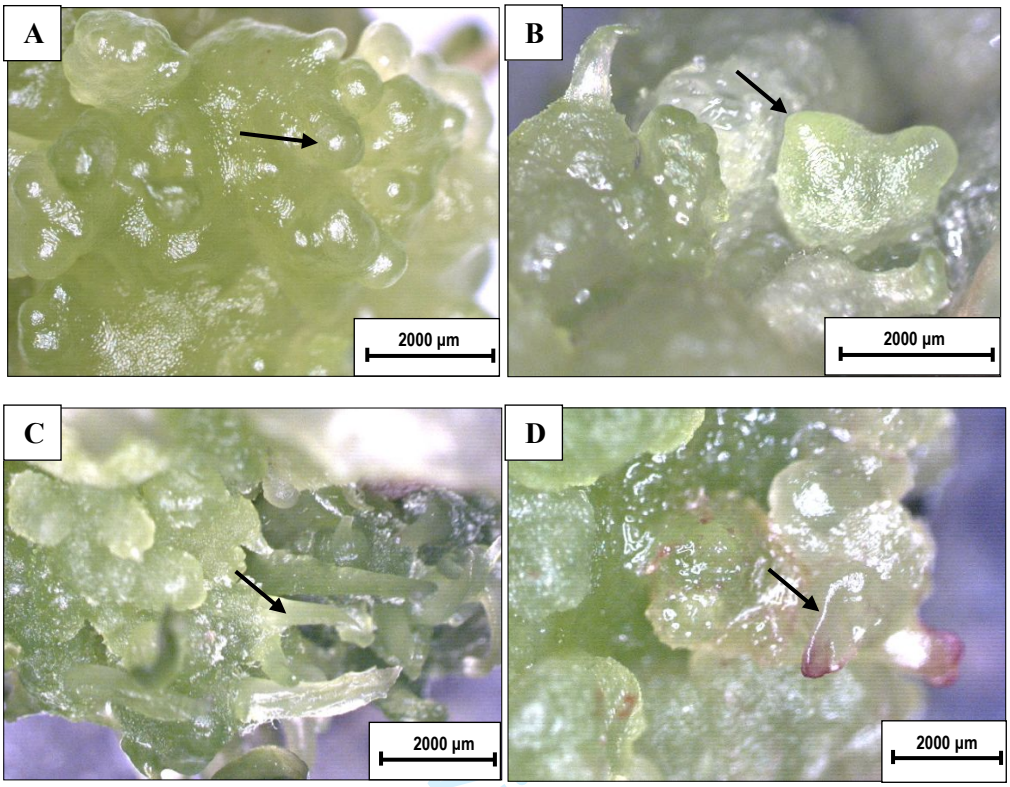

Figure 2. Different stages of somatic embryo development observed on leaf and root explants of *T. officinale* cultured on MS medium supplemented with BAP. (A) Globular structure (arrow), (B) Heart shape (arrow), (C) Torpedo structure (arrow), (D) Cotyledonary structure (arrow).

Figure 3. Acclimatized *in vitro* plantlets of *T. officinale* in the growth chamber after four weeks. Scale bars represent 5 cm.

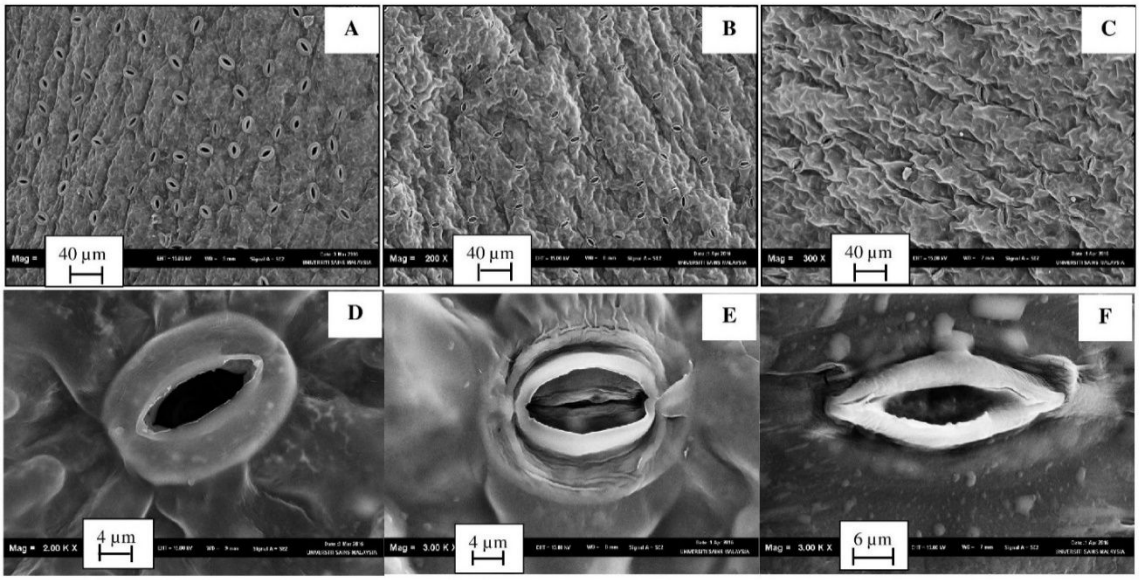

Figure 4. View of abaxial leaf surface and stomata of *T. officinale.* (A) and (D) *in vitro* plant, (B) and (E) acclimatized at 20 ± 2 °C, (C) and (F) acclimatized at 30 ± 2 °C.