African Journal of Biotechnology Vol. 8 (14), pp. 3239-3243, 20 July, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Influence of explanting season on *in vitro* multiplication of the medicinal herb, *Tridax procumbens* L.

C. P. Malik* and Chitra Wadhwani

School of Life Sciences, Jaipur National University, Jaipur, India.

Accepted 21 May, 2009

A protocol for complete regeneration of *Tridax procumbens*, a medicinally important plant, was accomplished through *in vitro* culturing. From nodal segments explants, high percentage of bud break and multiple shoot formation was induced between July and September on MS medium supplemented with BAP (1 mg L^{-1}). Rooting of the excised shoots from secondary cultures was excellent on half-strength MS medium having 1 mg L^{-1} IBA. Micropropagated plants when transplanted on garden soil flowered after 6 - 8 weeks. Explants sampled during December had low percentage of bud break and less shoot number per explant. Epicuticular wax (EW) and cuticular transpiration varied in the leaves collected during July and December; while under *in vitro* conditions, no marked differences were observed in regards to the 2 parameters. The epicuticular contents correlated with the rate of cuticular transpiration. The adaptive significance of EW during December is discussed.

Key words: *Tridax procumbens*, medicinal plant, micro propagation, explanting season, epicuticular wax.

INTRODUCTION

Tridax procumbens L, also known as Mexican daisy (Coatbuttons), though native of tropical America is naturalized in Asia. It is hardy, perennial, procumbent herb (Asteraceae). The plant is valued for its divergent pharmaceutical properties. The leaf juice has antiseptic, insecticidal and parasiticidal properties. It is used to check hemorrhage from cuts and wounds. The leaf juice is also used to check bruises and wounds. The leaves are also used in dysentery and diarrhea and also for preventing premature hair fall. The species is widely used in traditional medicine in India and is in great demand in the Indian pharmaceutical industry.

An aqueous extract of this plant also has marked depressant action on respiration. Earlier workers have already reported the presence of dexamethasone luteolin, glucoluteolin, *b*-sitosterol and quercetin in this plant (Subramanian et al., 1968; Reddy et al., 2006). The plant harbours immense medicinal potential. It is used to cure hepatitis. Its extract is used to increase immune inflammatory reactions, such as increase in phagocytic index, leucocyte count and antibody secret- ing cells. It has been shown to exhibit dexamethasone effects on wound contraction and granulation. *Tridax* develops granulation tissue in rats. It also affects lysyl oxidase activity. The extracts of *T. procumbens* have been reported to have various pharmacological effects, anti-microbial activity against both gram positive and gram negative bacteria and stimulate wound healing. Flavones, glycosides, polysaccharides and monosaccharides have been isolated from the leaves of the plant.

The species is widely exploited and its distribution has been declining over the years. It is not possible to micropropagate it through vegetative means, for example cuttings. Propagation through seeds is likely to cause variations. Recently methods have been developed for its *ex situ* conservation through micropropagation (Saini et al., 2008). The present studies were undertaken to investigate the influence of explanting season on *in vitro* multiplication of this plant.

The importance of cuticular wax for survival during stress has already been reported (Bernstein, 1975; Malik et al., 2008). During winter months plants exhibit slow growth and change the color of the leaves, that is, become hardy and rough. However, under tissue culture conditions, leaves remain thin and light green.

The objectives of the present investigations was to compare the response of various explants (apical bud, young leaves, nodal segments with and without axillary

*Corresponding author. E-mail:cpm_malik@yahoo.com.

Table 1. Response of apical bud and nodal explants of *T. procumbens* cultured on MS medium supplemented with different concentrations of BAP and meso-inositol. Observations were made after 10 days after culturing.

Days after inoculation (DAI)	BAP (1.0 mg L^{-1}) + Meso-inositol (100 mg L^{-1})		BAP (2.0 mg L^{-1}) + Meso-inositol (200 mg L^{-1})	
	Apical bud	Node	Apical bud	Node
1	No response	No response	No response	No response
2	No response	No response	Darkening	Darkening
3	Darkening	No response	Brownish black callus	Brownish black callus
4	Darkening	Darkening	Brownish black callus	Leaflet like structure
5	Brownish black callus	Darkening	Leaflet like structure	Leaf arises
6	Blackish callus	Brownish black callus	Leaf arises	Leaves arise
7	Leaflet like structure	Blackish callus	Increase in leaf size	Increase in leaf size
8	Leaf arises	Leaflet like structure	Increase in leaf size	Increase in leaf size
12	Leaves arises	Leaf arises	Formation of new shoots	Formation of new shoots

buds, internodes) and i.) to develop rapid and reliable a protocol for micropropagation of *T. procumbens* for use in genetic transformation, ii.) study adaptive changes in cuticular transpiration, iii.) compare the amount of epicuticular wax in plants under tissue culture conditions and field conditions, and iv.) evaluate the relationship between cuticular transpiration and amount of epicuticular wax.

MATERIALS AND METHODS

Sampling plant material

Healthy nodal explants (0.4 - 0.6 cm) having single dormant axillary bud was sampled from plants of *T. procumbens* L (Compositae) growing in the University Campus near the Institute of Pharmaceutical Sciences. The collections were made in 2 seasons: July-September and November - December, 2008

The explants were repeatedly washed with running tap water for 30 min and then treated with Laboline (4%) and 6% (v/v) sodium hypochlorite for 2 -3 min. Thereafter, they were washed with distilled water and surface sterilized with 0.1% mercuric chloride solution for 2 min. The explants were then rinsed with distilled water repeatedly.

Basal medium and cultural conditions

In the present studies modified Murashige and Skoog (1962) medium (MS) was used. The medium was supplemented with meso-inositol (00 mg L^{-1}, w/v) and sucrose (3% w/v). This medium was further supplemented with 0.5 - 4.0 mg L^{-1} KIN/BAP. The pH of the culture medium was adjusted to 5.8 and then gelled with 0.8% agar. The 30 ml medium thus prepared was dispensed in screw capped bottles. These were autoclaved at 104 kPa at 121°C for 20 min. The explants were surface sterilized and placed vertically on the culture medium. 5-6 explants were placed per jar. The cultures were maintained at 25°C under 16/8 h photoperiod with irradiance provided by cool white fluorescent tubes. The relative humidity was 60-68%. The light intensity was maintained at 40 umol m^{-2} S^{-1}.

Shoots multiplication

Primary shoots were formed after 3 weeks and these were isolated and cut into single node pieces and were denudated of leaves. The nodal segments with axillary bud were cultured on MS medium containing 1 mg L^{-1} BAP for multiplication.

Rooting of shoots

Shoots bearing 4-5 leaves were taken from secondary cultures and transferred to MS medium (½ strength) with sucrose (2%) and agar (0.7%). The medium was supplemented with 1.5 mg L^{-1} IBA.

Determination of contents of epicuticular wax and cuticular transpiration

Colorimetric methods were used for the estimation of contents of epicuticular wax and its contents by the methods of Ebercon et al. (1977), Voleti and Rajagopal (1991), Malik and Singh (1994) and Malik et al. (2008).

25 discs (16 cm^2) or 2 g of leaf material each were taken for the estimation of wax. Purified wax was used as a standard and contents were compared with it. Wax components were quantified through TLC on silica gel-G plates using the method of Holloway and Baker (1968) and as described by Malik and Singh (1994). The silica gel plates were activated at high temperature and spots were developed with benzene solvent. Plates were sprayed with 5% $K_2Cr_2O_7$ in 40% H_2SO_4 and heated at 150°C. The components were individually identified.

The method of Barnstein et al. (1978) was used to quantify cuticular transpiration. The experimental procedure was followed by at least 3-4 times. For comparison leaves were collected from the field plants during the months of September and then December.

RESULTS AND DISCUSSION

Several media were used with varied hormonal concentrations for the initiation of multiple shoot cultures in *T. procumbens.* Among the different explants tried (young leaves, nodal (with and without axillary buds), apical bud and internodal segments), only nodal explants with single dormant axillary bud responded positively 10 days after inoculation. Among the media tested for different stages, highest shoot development (%) and number of shoots per explant were highest with 2 mg L^{-1} KIN. This concentration caused maximum shoot length as well. BAP was most effective at l mg L^{-1}. The data set in Table 1 provi-

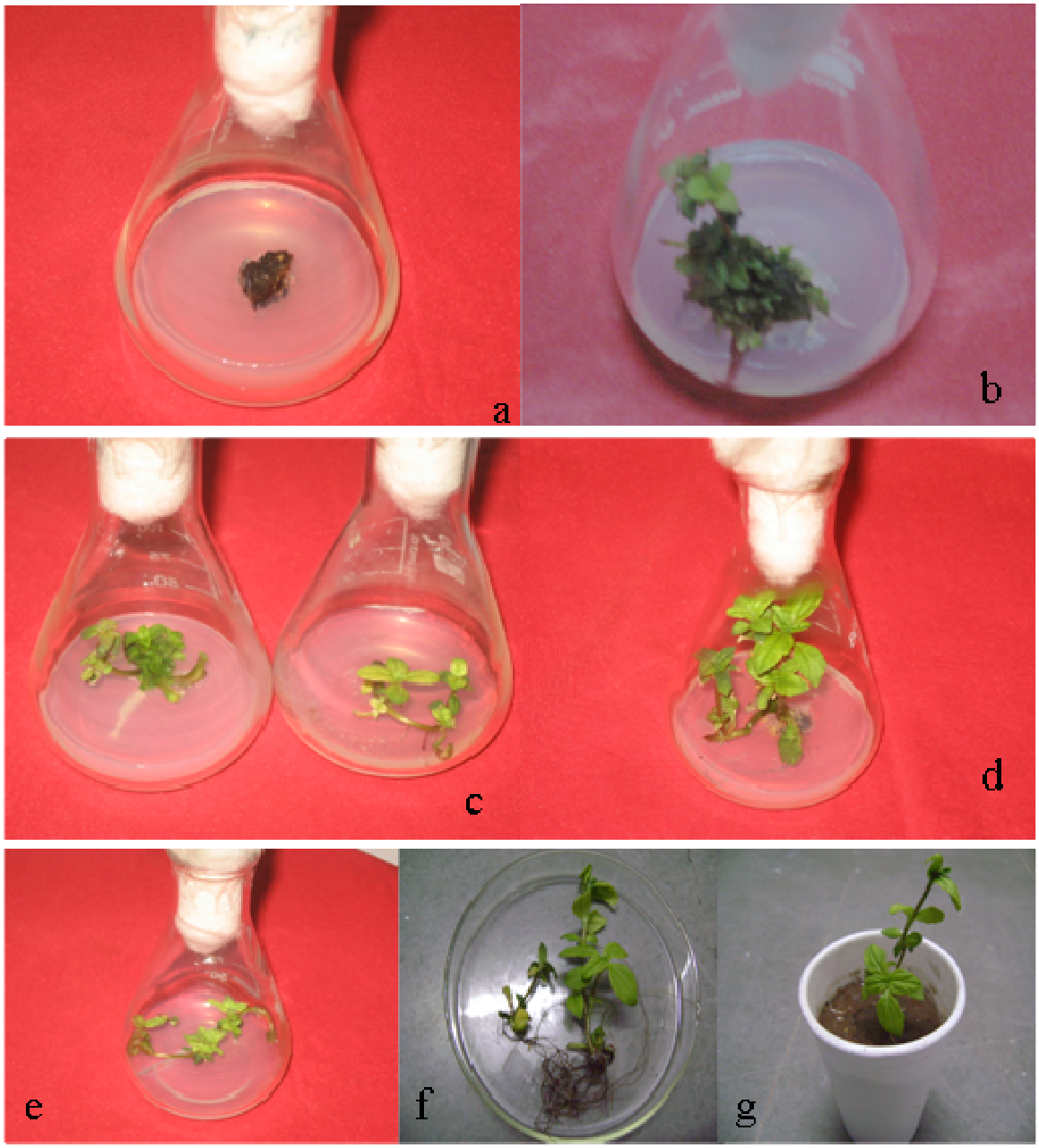

Figure 1a-g. *In vitro* multiplication in *Tridax procumbens* (a) Induction of callus from nodal explant, on MS medium supplemented with BAP (2 mg L-1+ mesoinositol 100 mg L-1) (b) callus with shoot primordial (c) shoot tip with leaves and roots after three weeks (d) shoots (e) A flowering shoot (f) Flowering shoot with copious roots (g) Transfer of plantlets to soil.

des detailed observations on the 3 parameters. Callusing occurred on MS basal medium supplemented with 1 mg L^{-1} of BAP (Figure 1). Later leaflets arose from the well developed callus. When BAP was supplemented with 100 mg L^{-1} myoinsitol.

In another experiment a combination of 2, 4-D (2 mg L^{-1}+ .5 mg L^{-1} KIN/BAP) caused callusing from nodal segments without axillary buds (Figure 1).

When MS medium supplemented with GA_3 was used axillary buds failed to sprout and the hormone also reduced bud break and number of shoots per explant even after 4 weeks of inoculation. When the concentration of GA_3 was enhanced it had negative influence on shoot development and also on number of shoots per explant. With a combination of BAP 1 mg L^{-1} + GA_3, bud break was rapid and it also promoted the frequency of bud break. The number of shoots nodal explant also enhanced. Sometimes roots developed with KIN/BAP + 2, 4-D.

Table 2. Morphogenetic response of nodal explants of *T. procumbens* cultured on MS medium supplemented with different concentrations of BAP/KIN, GA_3.

Treatment (mg L^{-1})	% Shoot development	Shoot number/explant
0.25 KIN	26.32	1.0
0.5	38.98	1.50
1.0	54.76	1.78
2.0	59.89	2.0
0	36.76	1.2
0.25 BAP	43.97	1.29
0.5	59.85	1.98
1.0	86.72	4.08
2.0	70.89	3.2
5.0	28.78	1.32
1.0 BAP + GA_3 .2	44.72	1.20
1.0 BAP + GA_3 .3	26.90	1.0

Table 3. Rate of cuticular transpiration in leaves from field sampled and tissue cultured seedlings (CT, mg H_2O cm^{-2} hr^{-1}) in July and December 2008 months.

DAS	July	December
Field sampled	0.581	0.36
Tissued cultured	0.50	0.48

Shoot proliferation

The period of the year when the explants were sampled had profound influence on shoot proliferation. The nodal explants sampled during July responded to various concentrations of KIN and BAP. The detailed data are given in Table 2. As is evident of the 2 cytokinins used BAP was found more effective than KIN for producing multiple shoots. Of the various concentrations tried, 1.0 mg L^{-1} was found to be most effective. At this concentration the number of shoots per explant was also highest (Table 2).

From each axillary bud single shoot emerged within 2 weeks and the shoot attained a length of 5-6 cm in 3 weeks time. When the concentration of BAP was increased beyond optimal level, there was suppression of sprouting.

When the explants were obtained during July-September, there was high bud break and produced maximum number of shoots. This was rainy season in northern India. When collected during December, there was least response. This was autumn season. During July collection % bud break was 85% whereas during December it was below 50%. Similarly number of shoots per explant was high during July (4.9) compared with December (2.4).

Half-salt strength MS medium devoid of any PGR failed to produce roots in the excised shoots from secondary cultures even after 6 weeks of culturing. We tried 3 auxins (IAA, IBA and NAA: 05, 1.0, 2.0 mg L^{-1}). Of these 1.0 mg L^{-1} IBA produced nearly 85% rooting. With NAA, no roots were induced but instead callus-like nodules were produced.

Acclimatization and transfer to soil

Plantlets having 4-5 fully expanded leaves and well developed roots were hardened in controlled growth chamber and then gradually shifted to outside environments. The plantlets were transferred to garden soil and full sunshine. Some of the plantslets flowered after 6 weeks and resembled the plants under natural conditions (Figure 1).

Epicuticular wax

The data given in Table 3 shows increase in total wax content with increasing months in field collected leaves. Based on spot density primary and secondary alcohols and aldehydes were identified and found to be high. In the leaves collected from tissue cultured plants (both seasons), was increase occured (Table 4) with progressive sub culturing. In both leaves from field and cultured conditions, wax contents were maximum in December. However, the contents of OH-B-diketones did not show any significant variation between the two.

Table 3 includes data on cuticular transpiration in cultured and field sampled conditions. In tissued cultured seedlings rate of transpiration was comparable to the field sampled leaves in July. But it reduced in December. December months experience low temperature (10-20°C), reduction in relative humidity. Hence accumulation of epicuticular wax possibly is an adaptive mechanism in *Tridax* to meet the adverse conditions under field conditions.

DISCUSSION

The promotory effect of BAP added to MS medium on bud break and multiple shoot formation in *T. procumbens* is comparable to the reports published in other medicinal plants (Debnath et al., 2006; Bhat et al., 1995; Pattnaik and Chand, 1996). We report suppression of sprouting with higher concentrations of BAP. Earlier, Pattnaik and Chand (1996) made similar observations in *Ocimum basilicum*. Though it was not evaluated here, but synergistic effect of different combinations of PGR has been reported and these are BAP + IAA (Sudha and Seeni, 1994), BAP + IBA (Lal and Ahuja, 1989) and BAP + 2,4-D (Sen and Sharma, 1991).

In our studies environmental factors/different seasons had a profound effect on shoot-bud differentiation from

Table 4. Contents of epicuticular wax with its components in leaves from feld sampled and tissued cultured seedlings in *T. procumbens.*

Sample	wax content		FA	OH-β-diketones	P alcohols	S alcohols	β-diketones	aldehydes
	(mg g FW)	(μg/cm^2)						
Tissued cultured								
Sep	1 7.64	1 54	+	-	+	v Lit	+	v Lit
Dec	17.00	158	+	-	+	v Lit	+	
Field sampled								
July	11.76	197	+	+	+	+	+	+
Dec	2 3.96	218	+	+	++	++	+	+

++ = High, + = low, - = absent, and v Lit = very little.

explants secured from nodal segments. Thus, July sampled explants gave better results than December explants.

The level of transpiration in *Tridax* leaves correlated with the amount of epicuticular wax and reduced rates of cuticular transpiration. In fact a linear relationship between epicuticular wax and contents of cuticular transpiration correlated with the surrounding temperature. We infer that β-diketones may be an indicator of glaucousness of leaves. A positive correlation was made out between reduced cuticular transpiration and high contents of primary and secondary alcohols and aldehydes.

Reduction in water loss provides distinct advantage to the seedling subjected to low temperature and accumulation of epicuticular wax might provide an adaptive system to *Tridax* plants to withstand adverse environment during extended periods of different stresses (Rao et al., 1981; Malik et al., 2008) conditions. Several studies have shown significance of enhancement of epicuticular wax and reduced rates of cuticular transpiration. During December month there was low temperature and reduced humidity under field conditions and these possibly affected the cuticle development and hence epicuticular wax accumulation. The unaltered conditions under tissued cultured conditions explain existence of uniform light intensity, temperature and humidity.

Under natural conditions, besides, primary and secondary alcohols, aldehydes also enhanced. A specific relationship was observed between transpiration rate and deposition of epicuticular wax.

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