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In vitro Plant Regeneration from Different Leaf Segments of *Verbesina encelioides* and Correlation with Endogenous Level of IAA

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Abstract

Variable leaf segments of *Verbesina encelioides* exhibited different responses when cultured on MS supplemented with 0.5 mg/l BAP. With the addition of IAA/NAA to the basal medium completely inhibited the formation and growth of callus. Endogenous content of IAA in the different segments of the leaf when assayed revealed that middle segment had highest level of IAA and apical and basal segments contained lowest contents. A specific correlation existed between the segment and callus formation.

Introduction

Verbesina is a genus of the sunflower family (Asteraceae) which contains over 60 species and is one of the largest families in the world**.** *Verbesina encelioides* (American dog weed, butter daisy, crown-beard, golden crown-beard or South African daisy) is native to the United States and Mexico and naturalized elsewhere. Seeds (achenes) are greyish brown, flattened, and broadly winged along margins. Long periods of seed dormancy and high germination rates are reported.

Chromosome number is reported as 2n = 34 (Vassileevska et al. 2002, Vassileevska 2005). There are many reports of its use in folk medicine as analgesic, emetic, febrifuge, insecticide and anti-inflammatory. It is even used to treat cancer, gastrointestinal disturbances, skin ailments, and snake bite. The plant is primarily an anti-inflammatory for redness and swelling of the orifices. The paste of *Verbesina* is applied directly to hemorrhoids, labial inflammations and sore gums. A hot cup of *Verbesina* tea is reported to bring down fever induces copious sweating, relaxation, and a mild laxative effect.

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Verbesina produces a range of eudesmane sesquiterpenes with cinnamate or a derived ester group, of which, a- and b-verbesinolcoumarates were the first reported examples. Several species are also source of elemanolides, diterpenes, flavonoids and biological active guanidines as galegine, the toxic principle of *V. encelioides* Benth. (Amaro-Lous et al. 2002)**.** *Verbesina* exhibits rapid seedling vegetative and reproductive growth. Seed germination under ideal conditions is high and can occur in different kind of soils except in gravel. It is significantly suppressed under drought and water logged conditions.

Verbesina encelioides is one such plant species. It is a drought-tolerant plant, requiring only monthly watering. In India, flowering is observed from August through October. Seeds are easily dispersed by light winds.

Though not widely treated ornamentally, there are a few companies in the Southwestern United States (specifically in New Mexico and Texas) that promote its planting due to its fast growing abilities, bright color of flowers, and having drought resistant qualities (Kaul and Mangal 1987). Additionally, the Arizona Department of Water Resources has this species listed on its "Official regulatory list of low water use and drought tolerant plants" as a recommended plant for landscaping usage due to its low water requirements.

The chief intent of the present study was the development of *in vitro* regeneration protocol which could be used fruitfully for *in vitro* conservation of this species. Though *Verbesina encelioides* is reported as recalcitrant plant in tissue culture, the present studies demonstrate that nodal explants and young leaves have high morphogenic potential for *in vitro* propagation. There have been attempts to perform *in vitro* propagation (Jain et al. 2008, Jain et al. 2009) but have failed to secure plantlets.

In most available studies combination of BAP + IAA or NAA yield friable green nodular callus from leaves (Palanivel et al. 2009). During micro propagation present authors observed that when leaves were plated on MS supplemented with BAP and then augmented with IAA or NAA no callus formation was observed. Thereafter they attempted to evaluate regeneration potential of different segments of a young leaf, from apex (A), middle (M) and basal (B) parts separately. The callus growth from each segment, with and without auxin, was recorded. The present investigation describes the role of different segments in callus differentiation when cultured on MS basal medium supplemented with 0.5 mg/l BAP.

Materials and Methods

For *in vitro* culture young, fresh rejuvenated leaves (1.5 cm) were collected from healthy field grow plants of *Verbesinia encelioides* (Heliantheae; Asteraceae) Jaipur National University campus, Jagatpura, Jaipur. Each leaf was divided into

three segments (apical A, middle M and basal B) and separately used as explants. The segments were classified in descending order from the leaf apex. The plants grow throughout the year though profuse growth is during October to June. Freshly collected seeds (October-December) were surface sterilized and germinated in a conical flask containing MS. Hypocotyls, axillary bud, immature leaves of seven days old seedlings were used as explants. The explants (leaf segments) were repeatedly washed with running tap water for 15 min and then treated with Laboline (4%) and 6% (v/v) sodium hypochlorite for 5 - 6 min. Thereafter, they were washed with distilled water and surface sterilized with 0.1% $HgCl_2$ solution for 5 min. The explants were then rinsed with distilled water repeatedly.

The individual segments used as explants were cultured on MS salts supplemented with phytohormone. The nutrient medium consisted of major and minor salts B5 vitamins (Gamborg et al. 1968), 3.0 % (w/v) sucrose and 0.8 % (W/V) agar. The pH of the culture media was adjusted to 5.8 before autoclaving (104 kPa) for 20 min at 121ºC and then gelled with 0.8% agar. The explants from the three sources were surface sterilized and placed vertically on the culture medium. 5-6 explants of the same type were placed per jar. The cultures were maintained at 20°C under 16/8 hr photoperiod with irradiance provided by cool white fluorescent tubes. The relative humidity was 60 - 68%. The light intensity was maintained at 40 μmol/m^2/s. After two weeks the regeneration potential of three types of segments were examined and then transferred onto fresh media.

Estimation of IAA was done by the method of Tang and Bonner (1947). In this a leaf was subdivided in three segments (A, M, B) and each segment was sampled from many fresh leaves of comparable size, nearly 200 mg of material was collected. The sample was crushed in chilled methanol (80%) and filtered through Whatman No. 1 filter paper. The final volume of the extract was made to 10 ml with chilled methanol. To 3 ml of this extract 6 ml of Salkowaski's reagent was added and the OD was recorded at 530 nm after 25 - 30 min of adding the reagent. The endogenous level of different leaf segments was computed using the standard curve of IAA.

Results and Discussion

Explants of leaves produced calli efficiently about two weeks after plating on the medium devoid of any auxin but having various concentrations of BAP (0.4, 0.5, 1.0, 2.0 and 3.0 mg/l). Only at high concentration calli formation was high. The callus was nodular, green and compact (Fig. 1). It started rooting after three weeks of plating. The leaf calli expanded but failed to undergo embryogenesis. In another experiment when young rejuvenate leaves were inoculated on to MS supplemented with BAP (0.5 - 3 mg/l) + NAA or IAA (1 - 3 mg/l), no growth

response or callusing was formed. Correlation between different leaf segments and callus formation and IAA contents in the segments was made.

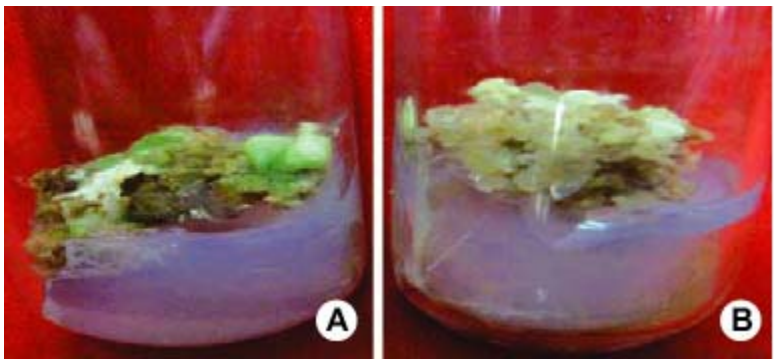

Fig. 1 A. Young leaf cultured on MS supplemented only with BAP (0.5 - 3 mg/l). B. Young leaf cultured on MS supplemented with BAP (0.5 - 3 mg/l) + IAA/ 2, 4 D (1 - 3 mg/l).

Young leaf and other explants were cultured on MS medium supplemented with BAP (0.5 - 3 mg/l) + IAA/ 2, 4 D (1-3 mg/l). There was poor callusing, which enlarged but gradually disintegrated. Young leaf (complete lamina) was used as explants and there was callus formation which enlarged, was friable and gradually turned brown, failed to differentiate but did set some roots. Subsequent experiments were done where different segments of the lamina were used as explants and the MS was devoid of any auxin. Fig. 2 shows response of different segments of a leaf used as explants for callus formation and rooting.

Apical part of the lamina (A) produced poor callus on MS supplemented with BAP (0.5 mg/l) and this callus mass enlarged and gradually disintegrated without differentiation. It failed to produce any roots. Fig. 2A shows callusing with basal segment having few roots. Fig. 2B shows green callus formations with middle (M) segment on MS + BAP without any auxin. Fig. 2C shows inoculation of axillary bud on MS + BAP (0.5 mg/l). From these studies it was assumed that endogenous level of IAA was possibly responsible for the differential behavior of different segments of lamia. In the first experiment where complete leaf was inoculated on the MS supplemented with IAA no growth was observed. Presumably young leaves contained abundant IAA and further addition of IAA to the MS resulted in growth inhibition. Soon after endogenous level of IAA was quantified using the method of Tang and Bonner (1947). Table 1 shows detailed data on auxin level and diamr of the segments.

The endogenous content of IAA in different leaf segments was quantified and was found in the range of 3 to 5 µg/ml leaf segment. When converted into 1.72×10^{-6} M and compared with the published values in actively growing tissues it appeared to be the comparable physiological concentration of auxin. From the

comparison of data with the existing values it is inferred that young leaves contain high content of endogenous IAA, therefore, using MS with BAP + IAA/2,4 D caused inhibition of growth and even callusing. Leaf segments, especially M segment, failed to elicit any response beyond callusing and rooting.

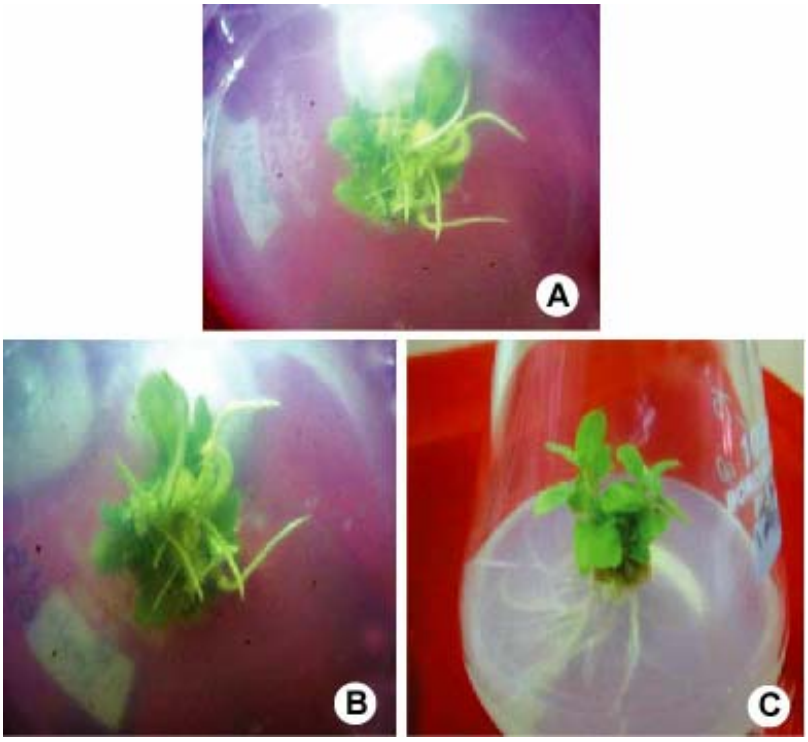

Fig. 2. Response of different segments of a leaf used as explants for callus formation and rooting. A. Callusing with basal segment, underwent swelling turned green and had few roots. B. shows green callus formations with middle segment on MS + BAP without any auxin. C. shows inoculation of axillary bud on MS + BAP (0.5 mg/l).

Young leaf or leaf segments were used successfully as explants to raise callus within two weeks of inoculation on MS supplemented with BAP (0.5 mg/l) successfully. Jain et al. (2008) established cell cultures from seeds on MS with or without IAA, NAA, Kn and BAP singly or in various combinations. Initiation of callus was observed after 20 days of inoculation and callus was successfully established on MS supplemented with 10 mg/l NAA and 0.4 mg/l Kn. The callus was whitish brown in color and friable in nature.

In most micropropagation investigations the workers use whole, rejuvenate immature leaves as explants (Venkatachalam et al. 1996, Kothari et al. 2010, Preetha et al. 2008, Malik and Wadhwani 2009). However, other explants are also used which include apical bud, axillary bud, ray florets, rachis, hypocotyls, seeds, embryos, embryonic axis, nodal segments, etc.

In the present studies, it was observed that the young leaf did not produce callus to the same extent as the middle segment of the leaf did in MS medium containing BAP + NAA/IAA and BAP without any auxin. Even this segment failed to respond when inoculated on BAP + auxin. Thus subsequent experiments revealed that the three segments contained variable level of auxin and that young leaves had high level of endogenous auxin to support callusing in the BAP alone.

Table 1. IAA content in three segments of leaves of *Verbesina encelioides*.

Leaf segment	endogenous content of IAA(μg/ml)	Area of each segment (cm)
A	4	0.48
M	5	1.18
B	3	0.65

Malik (1999) have discussed the role of auxin and cytokinin in cellular differentiation. Thus, for *in vitro* culture, growth of callus and plantlets is dependent on micronutrients and interaction of endogenous and exogenous supply of phytohormones. Present investigations clearly bring out that endogenous content of auxin was pivotal in supporting callusing and its further growth. Some workers have brought out that the balance between auxin and cytokinin was crucial for the formation of roots or shoots (Skoog and Miller 1957) and several factors contribute to the determination of endogenous content of auxin in a specific organ at a given time of growth. In general young leaves and shoot tips are active centers which abound in auxin. It is already shown here that young leaves are in an active metabolic growth phase having 1.7×10^{-4} to 1.71×10^{-6} M auxin and segment as M has high potential to callusing.

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