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Research Article

EFFECT OF GROWTH REGULATORS IN CALLUS INDUCTION, PLUMBAGIN CONTENT AND INDIRECT ORGANOGENESIS OF *PLUMBAGO ZEYLANICA*

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ABSTRACT

A high frequency and rapid protocol for callus regeneration has been developed in the medicinal plant *Plumbago zeylanica*. The present investigation is further aimed at determination of the plumbagin content in the callus and invivo plant.Profuse, compact callus was induced and proliferated from explants on MS medium fortified with 2,4-D or NAA (0.5 – 3 mg/l) alone and 2,4-D (0.5 – 4 mg/l) with BA or KIN (each at 0.1 mg/l , 0.5 mg/l). For shoot regeneration from callus MS medium supplemented with BA (mg/l) found to be the best medium when compared to other hormones tried. Best rooting of micro shoots obtained via callus regeneration observed on MS medium fortified with IBA (1.5 mg/l). The regenerated plants were acclimatized and then transferred to the field with 95% survival. The plumbagin content is comparatively higher in 2,4-D + BA hormonal combination or 2,4-D + KIN than in vivo condition.. The present study reports a successful indirect organogenesis protocol for the propagation of *Plumbago zeylanica* that helps in conservation and domestication.

Keywords: *Plumbago zeylanica* L., Callus regeneration, Indirect organogenesis and Acclimatization.

INTRODUCTION

Plumbago zeylanica belonging to the family Plumbaginaceae is highly valued for its medicinal properties due to the presence of an important alkaloid plumbagin – a naturally occurring naphthoquinone having high pharmaceutical value. The plant is a perennial shrub with sub-scandent stem distributed throughout India. The active principle – plumbagin distributed in the cells of secondary cortex and medullary rays of its roots [1] reported to possess anti- fungal, anti-bacterial, anti-tumor and anti- fertility properties [2,3]. The roots of *Plumbago zeylanica* used in many Ayurvedic preparations for the treatment of diseases like diarrhoea, dyspepsia, rheumatism, anasarca and piles. Approximately 60% of medicinal plants used in traditional medicines, roots are the principal source of drug preparation. The development of fast growing culture system can offer an opportunity for producing drugs from the roots in the laboratory without having to depend solely on field cultivation. Micropropagation can be considered as an important tool for the production of higher quality plant based medicines. Tissue culture studies in medicinal plants still hold key position in Biotechnology and will be the most promising biotechnological venture that can be exploited commercially for the production of pharmaceutical compounds. In view of this the present work aimed at developing an efficient protocol for plant regeneration from callus cultures of *Plumbago zeylanica* for the conservation of this valuable medicinal plant.

MATERIALS AND METHODS

Leaf and stem explants from healthy plants of *Plumbago zeylanica* were collected and thoroughly washed with water and surface sterilized with 0.1% $HgCl_2$ for 5 min followed by rinsing with double distilled water under aseptic condition on a laminar air flow. The medium comprised of macro and micro nutrients according to Murashige and Skoog(MS), with myo-inositol (100 mg/l), thiamine-HCl (0.1 mg/l), pyridoxine HCl (0.5 mg/l), nicotinic acid (0.5 mg/l), glycine (2 mg/l) and 3% sucrose as a carbon source gelled with 0.8% agar. MS medium is frequently used for micro propagation of large number of plants [4]. 2,4-D, NAA, BA, and KIN at different concentration either alone or in combination were used as plant growth regulators. The cultures were maintained at a temperature of 25 ± 2^{0}C under white fluorescent lamps of light intensity 3000 lux with a photo periodic regime of 16h light and 8 h dark cycles with 60-70% relative humidity. The p^H of the medium was adjusted to 5.8 before autoclaving at 121 ^{0}C and 15 lbs pressure for 20 min.

For callus induction explants were placed on MS medium supplemented with various plant growth regulators (2,4-D, NAA, BA, KIN) at different concentrations either alone or in combination. Further, MS fortified with BA or KIN (0.5-4mg/l) alone or in combination with IAA (0.1 mg/l, 0.4 mg/l) used for shoot regeneration. Shoots were excised and inoculated on MS medium with (0.5-2 mg/l) IAA, IBA and NAA separately for rhizogenesis. The shoots were also tested on hormone free full strength MS basal medium for root initiation.

Rooted plantlets were washed and potted in small plastic pots containing sterile vermiculate. They were covered with polythene bags to maintain humidity and watered regularly. After 4 weeks the polythene bags were removed and plantlets transplanted to earthen pots in a green house.

Determination of plumbagin production

In vivo and *in vitro* cultures were harvested and extracted under reflux with methanol. Plumbagin content was determined using a TLC-densitometric method [5]. Silica gel 60 F254 was used as a stationary phase and toluene:glacial acetic acid (99:1) was used as the mobile phase. The solvent system allowed a clear separation of plumbagin from other extract constituents. The identity of the compound was confirmed by the obtained Rf value, co-chromatogrphy with an authentic sample in at least three different solvent systems and UV absorption spectrum. Plumbagin content in each crude methanol extract was determined by TLC densitometry. Some 5.0 µl of the extracts was applied as the bands on silica gel 60 F254 TLC plates using an automatic spotter. After development in the above mentioned solvent system, absorbance was measured using a TLC densitometer. The conditions of the instrument were: UV detection, 254 nm; slit dimention 10.0 x 0.2 mm; monochromator band width 20 nm. The measurement was set to the mode of adsorption/reflection with scanning speed of 20 mm/sec. The area under the peaks of plumbagin was integrated and converted to concentration using its calibration curve. The calibration curve of plumbagin was established from an authentic sample at the concentration range of 0.062 - 1.0 mg/ml. and displayedas a linear equation of Y = 3507.66 X + 448.28 (r^2 = 0.9932).

Statistical Analysis

All experiments were carried out in a randomized design, ten replicates were raised for each treatment and experiments were repeated thrice. The growth of the plant was recorded every day. The data were analyzed statistically using one way analysis of variance (ANOVA), and the data means ± SD of at least three different experiments were represented and compared using Tukey-Kramer multiple comparisons test with the level of significant $P < 0.05$.

RESULTS AND DISCUSSION

Callus initiated from explants cultured on MS medium supplemented with all the combinations of 2,4-D or NAA alone and 2,4-D in conjunction with BA or KIN after 8 days of inoculation. Growth of callus increased significantly and covered the entire surface of the explants within 3 weeks. Callus is an unorganized mass of plant cells and its formation is controlled by growth regulating substances present in the medium [6]. Hormones, especially auxins were absolutely necessary for callus induction. Morphology of the callus varied with different plant growth regulators used in the medium. Among the different concentrations of 2,4-D tried highest response of callusing observed with 1mg/l on MS medium resulted in white friable callus. The need of 2,4-D alone in nutrient medium for the initiation of callus has been reported in some plant species [7-9]. But NAA alone showed least frequency of response for callus induction and produced dark brown callus which could not developed in to morphogenetic callus. The synergistic effect of 2,4-D with BA or KIN induced creamish white callus from explants (Table-1). Maximum proliferation of callus obtained when 1 mg/l 2,4-D along with 0.5 mg/l BA used. Combination of 2,4-D with BA for callus induction reported in Pineapple[10].

Table 1: Effect of 2,4-D in combination with cytokinins, BA/Kin for callus induction of *Plumbago zeylanica*

MS+ Hormones mg/l			Callus intensity (after 20 days)
2,4D	**BA**	**KIN**	
0.5	0.1	-	++
1	0.1	-	+++
2	0.1	-	++
3	0.1	-	+
4	0.1	-	+
0.5	0.5	-	++
1	0.5	-	++++
2	0.5	-	++
3	0.5	-	+
4	0.5	-	+
0.5	-	0.1	+
1	-	0.1	+++
2	-	0.1	++
3	-	0.1	+
4	-	0.1	+
0.5	-	0.5	++
1	-	0.5	+++
2	-	0.5	++
3	-	0.5	++
4	-	0.5	++

+,++,+++,++++ denotes increasing order of intensity.

To study the morphogenetic response, the calluses were subcultured on to regeneration media containing different concentration of BA or KIN alone or in combination with IAA. Addition of cytokinin in callus culture medium often enhances callus regeneration. Dark green shoot buds sprouted from the entire surface when calluses cultured in the medium fortified with BA or KIN. A high frequency of shoot regeneration observed in medium enriched with 1mg/l BA. Positive effect of BA alone on shoot regeneration from callus has been emphasized earlier by other authors [11,12]. Low rate of regeneration of shoot was marked in the medium having higher concentration of BA or KIN (Table-2). In contrast to the above mentioned results synergistic effects of auxins and cytokinins favored shoot regeneration from callus. BA(1mg/l) and IAA (0.1mg/l) showed highest regeneration ability (Table-2). These findings corroborated with that of other authors [13-15] reported that MS medium supplemented with BA and IAA enhance sprouting of shoot buds from callus. Shoot induction from callus is the function of cytokinin activity and relatively high ratio of cytokinin to auxin is mandatory in shoot initiation.

Table 2: Effect of growth regulators on shoot bud regeneration from callus

MS+ Hormones mg/l			Mean number of shoots after 30 days*
BA	**KIN**	**IAA**	
0.5	-	-	8.2±0.29
1	-	-	18.6±0.12
2	-	-	10.3±0.54
3	-	-	7.2±0.12
4	-	-	5.0±0.23
-	0.5	-	6.9±0.32
-	1	-	13.3±0.18
-	2	-	8.8±0.24
-	3	-	6.7±0.32
-	4	-	4.4±0.12
0.5	-	0.1	8.0±0.25
1	-	0.1	16.2±0.12
2	-	0.1	9.3±0.24
3	-	0.1	6.4±0.32
4	-	0.4	3.9±0.12

-	0.5	0.1	6.1±0.24
-	1	0.1	12.2±0.18
-	2	0.1	7.3±0.25
-	3	0.1	5.8±0.33
-	4	0.4	3.6±0.72

*Values represent mean ± SE of three replicates.

Roots developed on excised shoots grown in full strength MS medium with individual concentration of IAA, IBA, NAA. The 100% rooting observed within three weeks of transfer of regenerated shoots on 1.5 mg/l IBA containing medium. The present result coincide with the observation obtained by others [16,17]. The rooted plantlets transferred in to pots containing sterilized soil and sand in the ratio 1:1 for hardening. They were successfully established in the field with 95% survival frequency.

Callus cultures accumulated plumbagin at 0.016 ± 0.0030% DW. The cultures of *P.**z**eylanica* produced higher plumbagin concentration than those of *Drosera capensis* and *D. natalensis in vitro* cultivations [0.0004 % Fresh weight (FW)][18]. The concentration of plumbagin was, however, less than that of *Drosophyllum lusitanicum* suspension cultures (3.5% FW)[19, 20]. In addition, the present study found that the intact plants of the 3-year-old contain 0.32 - 1.16% DW of plumbagin. It was found that medium supplemented with 2,4-D (1 mg/l) + BA (0.5 mg/l) or 2,4-D (1 mg/l) + KIN (0.1 mg/l) was able to increase plumbagin production in the callus cultures. This study indicates that the appropriate hormonal combination with suitable explants is the way to succeed to increase the secondary metabolite production.

From all above observation it can be concluded that optimization for rapid callus induction and maximum shoot regeneration is a prerequisite in tissue culture technique. In the present study it was observed that *Plumbago zeylanica* can be very well propagated *in vitro* with fast rate through plant regeneration from callus cultures.

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