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Research Journal of Agricultural Sciences
An International Journal

P- ISSN: 0976-1675
E- ISSN: 2249-4538

Volume: 13
Issue: 03

Res. Jr. of Agril. Sci. (2022) 13: 704–709

CARAS

Res. Jr. of Agril. Sci. (May-Jun 2022)
13(3): 704–709

ISSN: 0976-1675 (P)
ISSN: 2249-4538 (E)

www.rjas.org

Full Length Research Article

An Investigation into the Potential of Developing a Method for Rapid Callus Induction and Micropropagation of *Wedelia chinensis* (Osbeck) Merr. – An Important Medicinal Herb

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Received: 06 Mar 2022 | Revised accepted: 10 May 2022 | Published online: 01 June 2022

A B S T R A C T

To develop an efficient method for fast callus induction and micropropagation of Wedelia *chinensis* (Osbeck) Merr. Surface sterilization of the explants was performed using varied concentrations of $HgCl_2$ and Tween20. Leaf and nodal explants of the species were used for callus and shoot induction respectively and the explants were reared on MS media augmented with various plant growth regulators (PGR) – auxin (1-Naphthaleneacetic acid (NAA) and Indole-3-butyric acid (IBA)) and cytokinin (6-Benzylaminopurine (BAP)). Sterilants (0.5% $HgCl_2$ + 0.1% Tween20) for 8 and 2 minutes respectively gave optimum results with 100% asepsis and the highest survival rate. Good results of induction of callus from leaf explants were found in MS media fortified with IBA (1 mg/l) + BAP (2 mg/l) which gave a quick response of callus initiation and also the escalated growth of the callus (1.092±0.08 g) within 60 days was more compared to other concentrations. Following an investigation, it was established that nodal explants when cultured on MS media augmented with IBA (1 mg/l) + BAP (2 mg/l) gave optimal multiple-shoot induction (10.9±0.18) and shoot growth (6.94±12 cm). For rooting of the well-established shoots, optimum results (7.4±15) were obtained when reared on half-strength MS media augmented with 2mg/l IBA. The well-established plantlets were weaned successfully in earthen pots with a 95% survival rate and the plants were phenotypically alike to the parent plant. The results signified that this protocol might be utilized for fast callus induction and successful plant regeneration from leaf and nodal explants respectively.

***Key words*:** *Wedelia chinensis* (Osbeck) Merr., Nodal explants, 6-Benzylaminopurine (BAP), Indole-3-butyric acid (IBA), 1-Naphthaleneacetic acid (NAA), Micropropagation

Wedelia chinensis (Osbeck) Merr. (Synonym Sphagneticola *calendulacea* (L.) Pruski is a member of the Asteraceae family and is often called as Birimagari in Sanskrit and Pilabhangra in Hindi [1]. It is a procumbent perennial herb with an unpleasant odour and the whole plant has a colossal medicinal value which is used in Unani, Siddha and ayurvedic systems of medicine [2]. It is considered to be highly antihepatotoxic and has several pharmacological activities such as hepatoprotective activities, antimicrobial activities, antioxidant activities, anticancer activities, androgen suppressing activities, wound healing activities, antiulcerogenic activities etc., [2]. The powdered form of root is used as a black dye, dying grey hair; tattooing and the leaves are useful in treating cough, cephalalgia and skin disorders [3]. The components isolated from this plant showed strong activity against damaged liver caused by biochemicals [4-5]. Furthermore, extracts of *W. chinensis* may have potential against anticonvulsant activity [6]. Also, bioactive compounds extracted from this plant demonstrated strong potential against prostate cancer cells [7]. Owing to the immense goodness of this species and therefore overexploitation from its wild habitat by some traditional practitioners, *W. chinensis* and other *Wedelia species* such as *W. calendulacea*, *W. trilobata* etc. are becoming rear medicinal herbs [8]. It is to be noted that by vegetative stem cutting and seeds germination, those herbs can be propagated but due to cross-pollination the species may induce some undesirable phytochemicals and characters [9]. So, the in-vitro culture of this type of herbal medicinal plant is very imperative to micro-propagate artificially. This is because *in-vitro* propagation using the technique of tissue culture, we would be able to produce precisely similar copies of plants, unalloyed metabolites needed and ineradicable preservation of the plant [10]. So, the sole purpose of our investigation was to establish an effective protocol for rapid

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callus induction and micropropagation of *W. chinensis* (Osbeck) Merr.

MATERIALS AND METHODS

For the investigation, 4-5 cm long apical parts of the shoot of *W. chinensis* were bought from the wild habitat inside the campus of the University of Science and Technology, Meghalaya. Running tap water was used for thoroughly washing the tips of the shoot (2-3 times) followed by repeated washing with distilled water. They were then treated with 0.1% Tween20 for 2-3 minutes followed by the transferring to a laminar air flow hood where further surface sterilization was done with diverse concentrations (0.1%-0.5%) of mercuric chloride for 5-8 minutes followed by rinsing several times with autoclaved distilled water to remove any micro amount of sterilizing agent. Aseptically and carefully, the nodal segment and the leaves were then dissected out and cut into proper pieces (1-1.5 cm) with a sterile surgical blade and utilized as explants. The nodal and leaf explants were then reared on MS basal medium containing sucrose (3% (w/v)) and the pH was maintained at 5.8±2 [11]. Agar powder (0.8% (w/v)) was used as a gelling agent and the complete MS media was sterilized by autoclaving for 15 minutes at 121°C at 15 lb pressure. The explants were inoculated in culture tubes containing 10ml of full-strength MS media boosted with varied concentrations and a combination of cytokinin (BAP) and auxin (IBA and NAA). Pre-sterilized glassware and instruments were used to carry out all the tasks. For each treatment, 10 replicates were used and the cultures were examined diurnally and the data and visual growth were noted and photographed and finally, the recorded data were put through statistical analysis for the computation of mean standard error ($\bar{x}$ ±SE). For the study, MS basal media without any growth regulators were employed to produce control cultures.

Leaf explants were utilized for the study of in-vitro callus induction whereas nodal explants were utilized for direct shoot regeneration. A variety of BAP, NAA and IAA concentrations and combinations was tested for optimal callusing and multiple shooting. The weight of the callus was recorded on the 30th day and the 60th day from the day of inoculation. The number of shoots in each explant and the height of the shoot were recorded after every 4-5 days interval. After 30 days of sub-culturing, individual shoots (3-5cm long) were subjected to in-vitro rooting after removal from the stock shoot culture. ½ MS basal media boosted with various concentrations and combinations of auxin (IBA and NAA) and activated charcoal were employed to test in-vitro rooting. 0.5g/l and 1.0g/l activated charcoal were also used to evaluate its effect on in-vitro rooting. The count of roots per explant was observed and recorded at every 2-3 days interval. All the in-vitro cultures were kept in the culture room under controlled conditions of temperature **(**26 ± 2°C) with cool white fluorescent tube light and provided with 16/8 hours light/dark photoperiod [12].

The complete plantlets obtained from nodal explants of *W. chinensis* with well-developed roots were taken out from the rooting media and washed with autoclaved distilled water; placed in plastic cups loaded with sand, peat and soil (1:1:1) and stored in a humid chamber for providing proper humidity. Finally, after adequate acclimatization, the established young plants were potted in earthen pots in natural environmental conditions with a 95% survival record.

RESULTS AND DISCUSSION

Surface sterilization of the explants used is one of the foremost and essential factors for the successful and contamination-free establishment of in-vitro cultures because the contamination reduces proliferation time and wastes re-culture time and efforts. The use of $HgCl_2$ and Tween20 is very common for surface sterilization of most of the plants used [13-15]. In the present investigation, the effects of a range of surface sterilants were evaluated for optimization of the contamination-free establishment of cultures. Optimum results with 100% asepsis and the highest survival rate were obtained when 0.5% $HgCl_2$ in combination with 0.1% tween20 for 8 minutes and 2 minutes respectively was used for surface sterilizing the explants (Table 1).

Table 1 Response to different surface sterilizing agents on leaf and nodal explants of *W. chinensis*

Sterilizing agent	Concentration (%)	Time of surface sterilization (min)	Observation (Percentage of contamination)
$HgCl_2$	0.1	8	100
$HgCl_2$	0.3	8	70
$HgCl_2$	0.5	8	10
Tween 20 + $HgCl_2$	0.1 + 0.3	2 + 8	30
Tween 20 + $HgCl_2$	0.1 + 0.5	2 + 8	0

Table 2 Effect of IBA, NAA and BAP on callus induction from leaf explants of *W. chinensis*

Hormones	Concentration (mg/l)	No. of days required for callus initiation	Percentage of callus induction (%)	Texture of callus	Colour of callus	Weight after 30 days from initiation (g)	Weight after 60 days from initiation (g)
Control	-	-	-	-	-	-	-
IBA	1	26	10	Friable	Yellow	Browning & dead	-
IBA	2	29	10	Friable	Yellow	Browning & dead	-
IBA	3	30	30	Friable	Yellow	Browning & dead	-
BAP + IBA	2+1	18	100	Friable	Yellow	0.061±0.056	1.092±0.08
BAP + IBA	2+2	27	60	Friable	Yellow	0.042±0.0052	0.073±0.0045
BAP + IBA	2+3	21	100	Friable	Yellow	0.032±0.02	0.025±0.17

Callus was induced from leaf explants of *W. chinensis* (Fig 1) on MS media fortified with various concentrations of IBA alone or in combination with BAP. Callogenic response was initiated within 3-4 weeks of inoculation in all the treatment concentrations excluding the treatment where MS media contained no growth hormone (Table 2). In the PGR free MS

media, the explants initially showed swelling, however, then turned brown and then died within 15-20 days of culture. Best results were observed with a maximum of 100% callus response and intended induction of callus within 18 days when the medium was fortified with IBA (1 mg/l) + BAP (2 mg/l). The calli obtained were yellowish in colour and friable in texture. All the cultures were maintained for about 60 days where the growth and weight of the calli were noted at 30 days and 60 days intervals.

The fresh weight of the callus in the media supplemented with IBA (1 mg/l) + BAP (2 mg/l) was 0.061±0.05 g after 30 days of callus induction which increased to 1.092±0.08 g after 60 days of culture. MS media boosted with IBA (3 mg/l) and BAP (2 mg/l) gave good results with 100% callus response and 0.032±0.02 g of callus after 30 days, however, the weight of the callus reduced to 0.025±0.07 after 60 days of culture. MS media containing only IBA gave 10-30% callus response within 26-30 days but then turned brown and subsequently died within 5-6 weeks of culture. Thus, in the present study, the combination of IBA and BAP was assessed to be best preferable for callus induction from leaf explants of *W. chinensis*. The synergetic effect of auxin and cytokinin for callus formation was also reported in *Achyranthes aspera* L. [16], broccoli [17], Ziziphora *tenuior* L. [18], and Celosia *argentea* (Var.) Cristata [19].

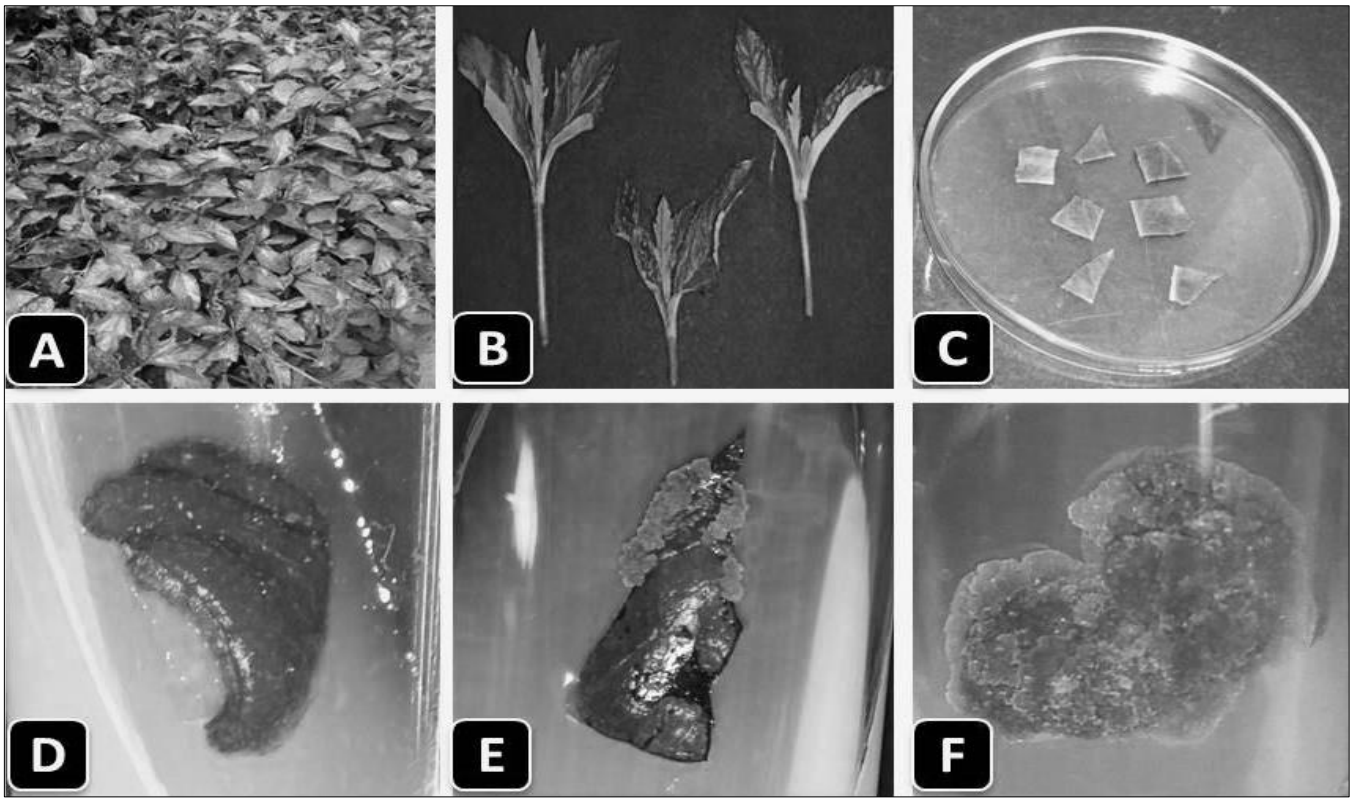

Fig 1 Different steps of callus induction from leaf explants of *Wedelia chinensis*
A = Habit, B = Shoot tips, C = Leaf explants, D = Inoculated explant, E = Callus induction at 30th day, F = Callus induction at 60th day

Table 3 Effect of IBA, NAA and BAP on in-vitro shooting from nodal explants of *W. chinensis*

Hormone	Concentration (mg/l)	No. of days required for shoot initiation	Percentage of shoot induction (%)	Mean no. of shoot/explant ($\bar{x}$ ±SE)	Mean length of shoot (cm) ($\bar{x}$ ±SE)
Control	-	-	-	-	-
NAA	1	8-10	65	2.2±0.41	2.64±20
NAA	2	8-10	55	1.2±0.31	1.86±27
NAA	3	8-15	65	1.0±0.12	2.9±16
IBA	1	9-10	72	2.2±0.23	1.54±11
IBA	2	10-15	65	1.5±0.31	3.54±28
IBA	3	8-10	72	2.1±0.18	3.54±18
BAP	2	8-10	50	3.6±0.12	**6.94±12**
NAA+BAP	1+2	9-10	75	4.5±0.25	4.32±24
NAA+BAP	2+2	8-10	75	3.5±0.22	5.38±15
NAA+BAP	3+2	8-10	78	3.6±0.23	2.36±11
IBA+BAP	**1+2**	**6-10**	**85**	**10.9±0.18**	6.24±16
IBA+BAP	2+2	9-10	85	3.8±0.21	5.76±23
IBA+BAP	3+2	10-21	75	2.3±0.32	3.16±20

Multiple shooting was observed using nodal explants of *W. chinensis* without the intervention of the callus phase (Fig 2A-E). It is known that multiple shoot buds develop effectively when both auxin and cytokinin is provided in the growth (MS) media [20-21]. In this study, multiple shoots were developed directly from the nodal explants on full strength

MS media fortified with different concentrations and a combination of cytokinin and auxin. Both frequency and magnitude of multiple shoot development and shoot elongation were greatly dependent on the concentration and combination of the auxin (IBA and NAA) and cytokinin (BAP) (Table 3). A maximal count of shoots per explant (10.9±0.18) was recorded when MS media was supplemented with IBA (1 mg/l) + BAP (2 mg/l) and multiple shoots were initiated within 6 days of culture with 85% of shoot induction. However, MS media containing NAA (1 mg/l) + BAP (2 mg/l) also showed effective supplement for multiple shoot induction but, the count of shoot per explant was less (4.5±0.25) compared to the former hormone combination. Moreover, days required for shoot induction (9 days) and percentage of multiple shoots induced (75%) were also noted to be less. A minimum number of shoots (1.0±0.12) were observed when the nodal explants were cultured in MS media containing NAA (3 mg/l). Multiple shoots were cultured for about a month in the same media combinations and after 30 days, elongated shoot lengths were recorded. Maximum elongated shoots (6.94±12 cm) were recorded in MS media boosted with BAP (2 mg/l) whereas minimum elongation of the shoot (1.54±11 cm) was noted in MS media augmented with IBA (1 mg/l). From the recorded data, it has been found that the best combination and concentration of PGR for multiple shootings did not provide the best shoot elongation in *W. chinensis*. Similar findings were also disclosed in the same species by Rahman and Bhand [22]. For multiple shoot induction, BAP in combination with IBA showed superior results compared to BAP alone and BAP in combination with NAA.

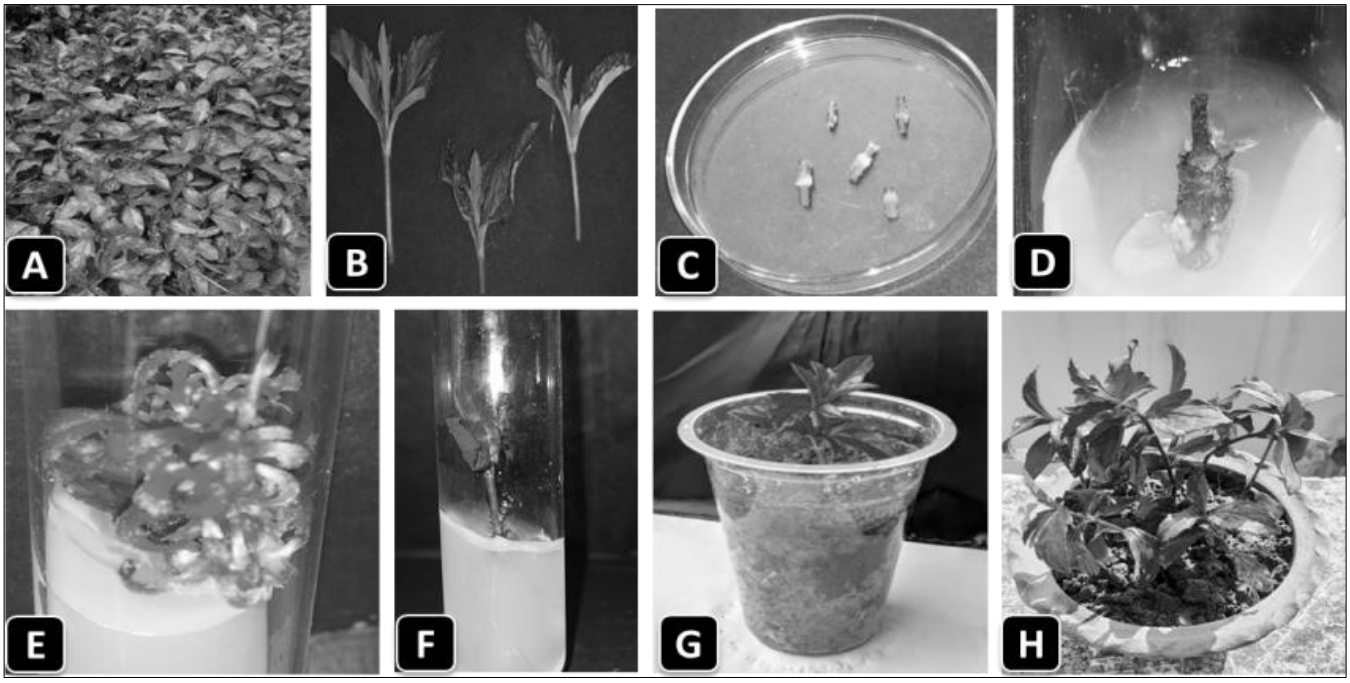

Fig 2 Different steps of micropropagation of *W. chinensis*
A = Habit, B = Shoot tips, C = Nodal explants, D = Inoculated explant, E = Multiple shooting after 30 days of inoculation, F = In-vitro rooting, G = Hardening of *W. chinensis* plantlets, H = Weaned plants in earthen pots in natural environment

Table 4 Effect of IBA, Activated charcoal and NAA supplemented media on rooting from in-vitro regenerated shoots of *W. chinensis*

Hormone	Concentration (mg/l)	No. of days required for root initiation	Percentage of root induction (%)	Mean no. of roots/explant ($\bar{x}$ ± SE)
Control	-	-	-	-
IBA	1.0	3-5	80	4.6±21
IBA	1.5	3-5	100	5.6±18
IBA	**2.0**	**3-4**	**100**	**7.4±15**
Activated Charcoal	0.5 % (w/v)	5-6	80	3.8±24
Activated Charcoal	1.0 % (w/v)	5-7	80	4.6±24
NAA	1.0	3-6	60	3.2±19
NAA	1.5	3-5	80	4.0±21
NAA	2.0	3-5	100	4.6±18

After about 30 days of culture, well-developed shoots (3-4 cm long) were isolated and cultured for in-vitro rooting on half-strength MS media boosted with different concentrations of activated charcoal, IBA and NAA (Table 4) (Fig 2F). Initiation of roots was observed in all the treatments within 1-2 weeks of subculture. Activated charcoal has been reported to have a positive effect on rooting in many plant species, e.g. *Sequoiadendron giganteum* [23], *Daucus carota* [24] and *Cattleya bicolor* [25]. Similarly, rooting was noticed on ½ MS media fortified with 0.5% and 1.0% activated charcoal, but was less effective than other PGR tested. Best results were obtained on ½ MS media augmented with IBA (2 mg/l) in which minimum time for root initiation (3-4 days) with 100% rooting response and having the maximum number of roots per explant (7.4±15) was observed. Conversely, the minimum count of roots (3.2±19) with 60% rooting response were obtained on ½ MS media when supplemented with NAA (1mg/l). Thus, from the recorded data, IBA has been found to be most effective in in-

vitro rooting of cultured shoots which was in agreement with the reports of Rahman and Bhand [22] in the same species [22]. The use of IBA as an effective auxin for in-vitro root induction has also been revealed in several medicinal plants, e.g. *Heracleum candicans* [20], *Solanum trilobatum* [26], *Plumbaga zeylanica* [27], *Tinospora cordifolia* [28], *Cassia alata* [29] etc.

Well-developed plantlets were obtained after about a month of subculture in the rooting medium. Before being transferred to the natural environment, these plantlets were taken out from the culture media, cleansed thoroughly and gently with distilled water. They were then cultivated in plastic cups filled with sand, soil and peat (1:1:1) and stored in a humid chamber for providing proper humidity and hardened through successive phases of acclimatization. The established plantlets were successfully transplanted in earthen pots and exposed to natural environmental conditions where the recorded survival rate was 95% (Fig 2 G-H).

CONCLUSION

From the results, it could be concluded that effectiveness of callus induction and in-vitro regeneration of *W. chinensis* depends on the concentrations and combinations of IBA and BAP. The protocol established could be an efficient method for micropropagation of *W. chinensis*. Furthermore, this protocol could also be employed for the genetic manipulation studies and improvement of *Wedelia* genotypes. Also, 1.092±0.08 g callus obtained within 8 weeks of culture suggests a fast callus growth which would be useful for metabolite production and extraction of pharmaceuticals from the callus without harvesting the plant from its natural habitat, thus, reducing its potential exploitation.

Acknowledgement

We are especially indebted to the University of Science and Technology, Meghalaya for providing material and laboratory support. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflicts of interest

The authors declare that they have no known competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.

***Ethics approval**: Not applicable.*

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