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RESEARCH ARTICLE

CALLUSING AND REGENERATION RESPONSE OF IN VITRO DERIVED LEAF EXPLANTS OF GERBERA JAMESONII

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ABSTRACT

Background: Gerbera hybrid seeds were obtained by half sib mating and the lines were raised in half strength MS medium. The response of *in vitro* leaf explants of *Gerbera jamesonii* to callusing and regeneration was recorded when cultured on full strength Murashige and Skoog's (1962) media supplemented with different concentrations of 2,4-D or BAP or IAA and Kinetin. Leaf explants cultured on MS medium fortified with equal concentration of BA (1 mg l^{-1}) and 2, 4-D (1 mg l^{-1}) produced green and greenish white granular callus within 25 days. But leaf explants cultured on MS medium supplemented with 2, 4-D produced yellowish calus. Plant regeneration was also found the earliest in the same media within 14 days.

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INTRODUCTION

The Barberton or Transvasal daisy, or simply gerbera, is a flower with increasing commercial significance. Although vegetative propagation is through divisions, plant multiplication by this method is much too slow to be commercially practicable. Divisions can achieve a 50 to 100 fold increase per year of a selected gerbera plant. In recent years, most of the varieties are multiplied through tissue culture. There has been increasing interest in tissue cultures as an alternative to asexual propagation of gerbera. This method enables a million fold expansions per year of a desired plant. Axillary shoot formation from excised capitulum explants (Pierik *et al*., 1973, 1974) or from shoot tips (Murashige *et al*., 1974) have been reported. Then it is possible to induce adventitious shoot formation on isolated young leaves derived from earlier developed axillary shoots. Plants regenerated from callus and adventitious shoots are required in mutation breeding as a tool for the production of solid mutants (Can *et al*., 2008). Cardosa and Silva (2013) observed the regeneration of adventitious shoots from leaf blades. The purpose of the present investigation was to study the response of *in vitro* derived leaf explants to callusing and regeneration.

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MATERIALS AND METHODS

The seeds obtained from the individual cross were surface sterilised, soaked in distilled water for 30 minutes with the addition of 1-2 drops of Tween-20 followed by 40% sodium chloride solution and gently agitated. The seeds were then rinsed three times with distilled water and then soaked in mercuric chloride (0.1%) for 3 to 4 minutes under laminar airflow. Finally, the seeds were again washed with autoclaved distilled water. Half MS medium (Murashige and Skoog, 1962) was used for inoculation of seeds. The following observations were recorded were as follows days taken for germination, days taken for first leaf emergence, days taken for rooting, number of leaves after 60 days, number of roots after 60 days, shoot length after 60 days. Individual *in vitro* plants were raised under well defined culture room conditions. Individual seeds from the cross were considered as a single line and hence basic statistical measures such as mean was applied. *In vitro* leaf explants from fourteen hybrid lines were used. The MS (1962) basal medium was used at full strength. Each basal medium was supplemented with different concentration of growth regulators in five treatments.

T_1 - MS and BA (1 mg l^{-1})
T_2 - MS and BA (1 mg l^{-1}) and IAA (1 mg l^{-1})
T_3 - MS and BA (1 mg l^{-1}) and 2,4-D (1 mg l^{-1})

T_4 - MS and Kinetin (5 mg l^{-1}) and IAA (1 mg l^{-1}) and 2,4-D (1 mg l^{-1})
T_5 - MS and BA (5 mg l^{-1}) and Kinetin (5 mg l^{-1}) and 2,4-D (1 mg l^{-1})

The pH of all media was adjusted to 5-6 before sterilization by the addition of 1N HCl or 1N NaoH as required. The containers having the medium were sterilized at 15 lbs/m^2 at 121°C for 20 minutes. The leaves with petiole from *in vitro* grown seedlings were used as explant. The leaves were cut into 4 to 5 mm in size, were placed in the culture bottles. Injury was made on the leaf before placing in the medium. Completely randomized design was followed with five replications. Observations were recorded on days taken for callus initiation, callus production, days to develop plantlets from the callus, days to form shoots and roots per callus clump.

RESULTS AND DISCUSSION

Least number of days for germination was observed in the line IIHR 1-1 with an average of 2.00 days. Whereas, IIHR 3-3 took the highest days (7.00) to germinate followed by IIHR 4-2 (6.00), compared to all the other lines (Table 1). The variation observed in the different genotypes might be attributed to their genetic make-up (Chobe *et al.,* 2010). The line IIHR 5-4 recorded earliest first leaf development (3.00 days) over other lines. The highest number of days taken for first leaf development (8.00) was recorded from the line IIHR 3-3. Addition of auxins together with cytokinin becomes essential for shoot induction and multiplication depending on the plant type. The right combination of auxin and cytokinin in the culture medium determined the effectiveness of micropropagation of gerbera.

Table 1. Response of *in vitro* raised seeds in half MS medium

Lines	Days taken for germination	Days taken for first leaf emergence	Days taken for first rooting	Number of leaves after 60 days	Number of roots after 60 days	Shoot length at 60 days
IIHR 1-1	2.00	4.00	3.00	5.00	3.00	6.00
IIHR 1-2	3.00	4.00	3.00	4.00	3.00	6.00
IIHR 1-3	4.00	6.00	4.00	4.00	3.00	6.00
IIHR 1-4	4.00	5.00	4.00	4.00	3.00	6.00
IIHR 1-5	3.00	5.00	3.00	3.00	4.00	5.00
IIHR 2-1	3.00	4.00	4.00	3.00	3.00	6.00
IIHR 2-2	3.00	6.00	4.00	4.00	5.00	6.00
IIHR 2-3	3.00	6.00	4.00	4.00	6.00	6.00
IIHR 2-4	4.00	6.00	5.00	5.00	5.00	6.00
IIHR 2-5	4.00	6.00	5.00	4.00	5.00	5.00
IIHR 3-1	4.00	5.00	5.00	5.00	5.00	6.00
IIHR 3-2	3.00	4.00	3.00	4.00	5.00	7.00
IIHR 3-3	7.00	8.00	8.00	3.00	4.00	6.00
IIHR 3-4	4.00	6.00	5.00	4.00	5.00	7.00
IIHR 3-5	5.00	6.00	5.00	6.00	5.00	4.00
IIHR 4-1	3.00	5.00	4.00	5.00	4.00	5.00
IIHR 4-2	6.00	6.00	5.00	4.00	5.00	5.00
IIHR 4-3	3.00	6.00	4.00	6.00	5.00	6.00
IIHR 4-4	3.00	5.00	4.00	6.00	5.00	6.00
IIHR 4-5	4.00	7.00	4.00	4.00	5.00	7.00
IIHR 5-1	3.00	5.00	4.00	5.00	5.00	6.00
IIHR 5-2	4.00	6.00	4.00	5.00	4.00	6.00
IIHR 5-4	3.00	3.00	3.00	4.00	4.00	8.00
IIHR 5-5	3.00	5.00	6.00	3.00	4.00	6.00
Mean	3.52	5.16	4.12	4.16	4.20	5.72

Table 2. Effect of different MS treatments on callus induction from leaf explants at 40 days

Lines	MS+2,4-D (1.8 mg l^{-1})	MS+2,4-D (2 mg l^{-1}) +BA (1 mg l^{-1})	MS
IIHR 1-1	++	++	0
IIHR 1-2	++	++	0
IIHR 1-3	+	+++	0
IIHR 1-4	++	++	0
IIHR 1-5	++	++	0
IIHR 2-1	+	++	0
IIHR 2-2	++	+++	0
IIHR 2-3	++	++	0
IIHR 2-4	++	++	0
IIHR 2-5	++	+++	0
IIHR 3-1	+	++	0
IIHR 3-2	++	+++	0
IIHR 3-3	+	+++	0
IIHR 3-4	++	+++	0
IIHR 3-5	++	+++	0
IIHR 4-1	0	0	0
IIHR 4-2	++	++	0
IIHR 4-3	++	++	0
IIHR 4-4	+++	++	0
IIHR 4-5	++	++	0
IIHR 5-1	0	0	0
IIHR 5-2	0	0	0
IIHR 5-4	++	++	0
IIHR 5-5	0	0	0

0: No callus, +: Poor growth of callus, ++: Moderate growth of callus, +++: Vigorous growth of callus

Table 3. Effect of the treatments on callus production and regeneration

Treatments	Days to initiate callus	Days to initiate plantlet from callus	Number of shoots/ clump	Number of roots/ clump
MS and BA (1 mg l^{-1})	26.62	14.75	3.50	0.12
MS and BA (1 mg l^{-1}) and IAA (1 mg l^{-1})	29.50	14.87	1.00	2.25
MS and BA (1 mg l^{-1}) and 2, 4-D (1 mg l^{-1})	25.87	13.75	3.12	1.12
MS and Kinetin (5 mg l^{-1}) and IAA (1 mg l^{-1}) and 2, 4 D (1 mg l^{-1})	29.62	22.00	0.75	0.87
MS and BA (5 mg l^{-1}) and Kinetin (5 mg l^{-1}) and 2, 4 D (1 mg l^{-1})	32.12	22.40	0.75	0.62
S. E.m±	0.18	0.33	0.10	0.15
C.D. at 1%	0.54	1.42	0.35	0.23

Table 4. Response of lines to callus formation and regeneration

Lines	Days to initiate callus	Days to initiate plantlet from callus	Number of shoots per clump	Number of roots per clump
IIHR 1-1	24.00	15.00	3.33	1.66
IIHR 1-2	28.00	17.00	2.33	2.00
IIHR 1-3	31.00	15.00	2.66	2.00
IIHR 2-1	32.00	14.00	2.66	2.00
IIHR 2-2	32.66	13.33	2.66	1.00
IIHR 2-5	30.00	17.00	3.00	2.00
IIHR 3-1	30.00	18.00	2.66	2.00
IIHR 3-2	24.66	20.00	2.00	1.00
IIHR 3-5	29.66	20.33	1.66	1.00
IIHR 4-1	29.33	20.00	2.00	1.66
IIHR 4-2	30.33	19.00	2.00	1.66
IIHR 4-4	29.33	20.66	2.00	1.66
IIHR 4-5	30.33	20.00	1.33	1.00
IIHR 5-4	30.33	19.66	1.33	1.66
S. E.m±	0.50	0.56	0.30	0.43
C.D. at 1%	1.45	1.63	0.89	1.30

Addition of strong auxin with BAP promoted better shoot formation compared to weak auxin (Pierik *et al.,* 1973). In Table 1 the lines IIHR 1-1, IIHR 1-2, IIHR 1-5, IIHR 3-2 and IIHR 5-4 took the least number of days (3.00 days) for rooting compared to other lines, however, IIHR 3-3 recorded maximum number of days for first rooting (8.00 days). IAA proved to be more efficient in produced maximum number of good quality, healthy and thick roots. Pagnussat *et al*. (2004) reported that IAA increases the number of roots through the development of meristematic tissues and regulation of cell differentiation. After the seeds germinated, the plantlets were sub cultured in a medium containing BAP (2 mg l^{-1}) along with IAA (1 mg l^{-1}). Because, the role of auxins and cytokinin in micropropagation is well known and the best morphogenetic response can be obtained from synergistic effect of compatible auxins and cytokinin combination (Aswath and Choudhary, 2001). The favorable effect of cytokinins on shoot meristem initiation, axillary bud bursting and multiple shoot production have been demonstrated by Pierik *et al.* (1975). The number of leaves after 60 days was found to be considerably increased in the line IIHR 3-5, IIHR 4-3 and IIHR 4-4 (6 leaves per plant) when subcultured (Table 1). In the present study it was noticed that several roots developed spontaneously from the *in vitro* grown shoots but the spontaneously developed roots were found to be inadequate for transplantation of the *in vitro* grown shoots to the soil. Therefore, separate root induction was necessary.The plants were transferred in a rooting medium containing half MS along with 1.5 mg l^{-1} IBA. Highest number of roots was obtained in the line IIHR 2-3. Aswath and Choudhary (2001) also reported maximum root induction and average number of roots per shoot when cultured on MS medium containing 1.5 mg l^{-1} IBA. Line IIHR 5-4 was significantly superior for shoot length (8.00 cm) over other lines, whereas, the line IIHR 3-5 had the lowest shoot length (4.00 cm) (Table 1).

Optimum concentration of growth regulators required varies with different cultivars every genotype had a specific range of optimum growth regulator concentration (Deepaja, 1999). The callus was initiated in all the cultures within 26-32 days of culturing, irrespective of the strength of the nutrient medium (Table 3). The growth regulator combination of 2,4-D with BAP was proved to be the best compared to other treatments in the basal nutrient medium. First callus was observed on the leaf explants cultured on MS medium supplemented with 1.0 mg l^{-1} 2,4-D + 1 mg l^{-1} BAP (25.87 days) produced the callus among all the treatments. 2,4-D with BAP definitely stimulated callus production whereas IAA with BAP though produced callus took a long time to initiate callus. The present result is also in confirmation with Aswath *et al.* (2003) and Kumar *et al*. (2004). After 60 days of inoculation, callus produced from the explants were scored visually and analyzed (Table 2). Explants cultured on full MS medium supplemented with lower levels of 2,4-D (2.0 mg l^{-1}) and BAP (1 mg l^{-1}) produced more amount of callus. There was no callus production on the basal medium and the leaf explants remained as it is. Growth regulator addition is a must to stimulate cell division. BAP is essentially required for the formation of callus. Kumar and Kanwar (2006) proved synergetic effect of BAP on gerbera. To conclude, 2,4-D and BAP addition to full strength MS basal medium produced the best callus, both quantitatively and qualitatively. However, there was difference in the type of callus produced. MS basal medium fortified with 2,4-D and BAP produced green and nodular callus and MS medium fortified with 2,4-D alone produced yellowish creamy callus in all the treatments irrespective of the varieties. This result is also in confirmation with Hasbullah *et al.,* 2008. The callus was observed under microscope for somatic embryogenesis validation. There was no vascular connection between the embryos and they were popping out from the clump, clearly indicated that they are somatic embryos. Naing *et al.* (2011)

also reported that somatic embryos were indirectly induced from leaf derived callus. Similar results were also reported by Huan *et al*. (2004). The plantlets were initiated from the callus with 13-22 days of culturing irrespective of strength of the nutritive medium (Table 3). Among the lines IIHR 2-2 took the least time of 13.33 days to form plantlet (Table 3), whereas, the combination of MS medium supplemented with BA (1 mg l^{-1}) and 2,4-D (1 mg l^{-1}) recorded earliest days to initiate plantlet from callus (13.75 days). This is in confirmation with studies of Martin (2004) who reported that even low concentration of 2,4-D influenced somatic embryogenesis when added to the culture medium.

The number of plantlets from the callus ranged from 1-4 (Table 3 and 4). Highest number of plants per clump (3.50) was recorded on MS and BA 1 mg l^{-1}. The line IIHR 1-1 produced 3.3 plantlets from each clump. BAP being the chemical analogue of cytokinin not only affect different phases of regeneration but also indicate cytokinin specificity for obtaining higher number of regenerants and shoots which is in corroboration with the findings of Aswath and Wazneen (2004). The treatment MS and BA (1mg l^{-1}) and IAA (1mg l^{-1}) produced highest number of roots (2.25). The lines IIHR 1-2, IIHR 1-3, IIHR 2-1, IIHR 2-5 and IIHR 3-1 were recorded with highest number of roots (2.00) per callus clump. This is in confirmation with Kumar *et al.,* 2004. This variability between the lines could be attributed to genotypic difference and their response to phytohormones. Further study of histology of cultured leaves is necessary to understand the cells and factors contributing towards regeneration. The production of adventitious shoots, which will be true to type to the mother plants, can be proposed as an alternative method of propagation. Competence of immature leaves for regeneration could also be exploited for creation of transgenics through *Agrobacterium* mediated genetic transformation, since it has been demonstrated that the juvenile tissue are genetically more susceptible for Agrobacterium.

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