PLANT REGENERATION FROM CELL SUSPENSIONS IN *Gerbera jamesonii* Bolus

Surinder Kumar* and Jitender Kumar Kanwar

Department of Biotechnology, University of Horticulture and Forestry
Solan – 173 230, H.P., INDIA

*Corresponding author: e-mail: skuhf07@yahoo.co.in

(Received July 3, 2007/Accepted September 12, 2007)

A B S T R A C T

Plants of *Gerbera jamesonii* were regenerated from cell suspension cultures of calli derived from leaf tissue explants. Callus induction was best when the medium contained either 1.5 or 2.0 mg l^{-1} 2,4-D. The percentage of calli that produced shoots and the number of shoots per callus were highest on MS medium supplemented with 1.0 mg l^{-1} BA. Calli cultured on medium containing 1.5 mg l^{-1} 2,4-D were highly friable and had a low regeneration potential. These calli were selected for cell suspension studies. Cell suspensions were started in liquid MS medium containing 1.5 mg l^{-1} 2,4-D. The suspensions were sub-cultured once a week to ensure maximum cell density. The cultures were allowed to form micro-colonies in liquid medium. These micro-colonies developed into calli when transferred to solid or semi-solid medium. The calli differentiated into adventitious shoots on MS medium supplemented with 1 mg l^{-1} BA. 70 to 80% of the plantlets derived from the shoots were still alive thirty days after they were transferred to pots.

Key words: pot gerbera, liquid medium, growth regulators, adventitious shoots

INTRODUCTION

Gerbera species are commonly grown as potted plants or for cut flowers. In this genus, genetic variability is relatively limited, which makes it difficult to breed for new flower colors and patterns. There is also limited genetic material that can be used to improve resistance to biotic and abiotic stress (Orlikowska et al., 1999).

Gerbera plants can be propagated either sexually or asexually. Until recently, they were usually propagated by dividing clumps. However, this technique is too slow for commercial purposes (Murashige et al., 1974). Nowadays, *in vitro* propagation is the preferred method (Reynoird et al., 1993; Aswath and Choudhary, 2002; Xi and Shi, 2003; Prasanth and Sekar, 2004; Kumar et al., 2004).

Gerbera plants have been regenerated from calli derived from a variety of explants (Zheng et al., 2002; Modh et al., 2002; Tyagi and Kothari, 2004; Ray et al., 2005; Kumar and Kanwar, 2006).

Adventitious shoots have been regenerated from the flower buds of *Gerbera* plants grown in the greenhouse (Pierik et al., 1975; Laliberte et al., 1985). Attempts at regenerating shoots using midrib explants were not successful (Pierik and Segers, 1973). In a study on the *G. jamesonii* cultivar 'Vulkan', shoots were regenerated *in vitro* using leaf blade explants (Hedtrich, 1985).

The aim of the present study was to determine the effect of various growth regulators on callus formation and shoot regeneration from explants of *Gerbera* leaf tissue. A new propagation protocol was also elaborated by which plants were also regenerated from cell suspensions cultures of the most friable of the calli.

MATERIAL AND METHODS

Callus culture

Pot gerbera (*Gerbera jamesonii* Bolus) was grown in the greenhouse at the Department of Biotechnology, University of Horticulture and Forestry, Solan (H.P.), India.

Leaves were collected, and the petioles were removed. The leaves were cut into small explants five to six millimeters in size under sterile conditions. The explants were surface sterilized with 0.1% $HgCl_2$ for three to four minutes, rinsed with sterile distilled water, and cultured on MS medium (Murashige and Skoog, 1962). The medium was supplemented with 30 g l^{-1} sucrose and solidified with 8 g l^{-1} agar. The pH of the medium was adjusted to 5.8 before autoclaving.

The following growth regulators were also added to the medium:

- IBA (indole-3-butyric acid): 1.0, 1.5 or 2.0 mg l^{-1};
- NAA (naphthaleneacetic acid): 1.0, 1.5 or 2.0 mg l^{-1};
- 2,4-D (dichlorophenoxyacetic acid): 1.0, 1.5 or 2.0 mg l^{-1};
- BA (benzyladenine): 1.0, 1.5, 2.0, 3.0 or 5.0 mg l^{-1}; or
- Kinetin: 1.0, 1.5, 2.0, 3.0 or 5.0 mg l^{-1}.

Each treatment was applied in three replications of ten explants each. The explants were cultured in a growth room at 25°C in the dark to encourage the formation and growth of callus. Explants cultured without growth regulators served as the control.

When the calli were one month old, those between four and six millimeters in diameter were transferred to a growth chamber and maintained under a 16 hour photoperiod under artificial light at an intensity of 100 µmol m^{-2} s^{-1}. Shoot regeneration was encouraged by culturing the calli on MS medium supplemented with 1 mg l^{-1} BA.

After thirty days, the following data were recorded: percentage of explants producing callus; callus growth; and callus type. After sixty days, the following data were recorded: percentage of calli producing shoots; and average number of shoots per callus.

All data were statistically elaborated using analysis of variance for a completely randomized design as described by Gomez and Gomez (1984).

Cell suspension culture

Twenty suspension cultures were started using the most friable calli derived from the leaf explants. Portions of callus weighing between 0.5 and 1.0 g were placed in 100 ml Erlenmeyer flasks containing 20 ml of liquid MS medium. The medium was supplemented with 1.5 mg l^{-1} 2,4-D. The suspensions were placed on a rotary platform shaker set at 100 rpm and kept at 25°C in the dark. The suspensions were subcultured once a week to ensure maximum cell density.

The suspensions were filtered through autoclaved double-layered muslin in order to separate cells from micro-calli and debris. The suspensions were regularly subcultured by transferring 5 ml of suspension into 20 ml of fresh liquid medium. Cell density was measured with a hemocytometer, and adjusted to between 10,000 and 30,000 cells per milliliter by adding more culture medium. The viability of isolated cells was confirmed by using Evan's blue dye. Freely suspended cells were examined under a light microscope and photographed for future reference.

2 ml of the suspension was added to 4 cm Petri dishes containing 10 ml of semisolid and solid MS medium supplemented with 1.5 mg l^{-1} 2,4-D. The Petri dishes were swirled to ensure that the cells were evenly distributed in the agar medium. The Petri dishes were then sealed with parafilm and incubated in the dark so that the cells could grow and differentiate. Calli were regenerated on MS medium supplemented with 1 mg l^{-1} BA.

Individual shoots were separated and rooted on MS medium supplemented with 1 mg l^{-1} BA and 0.1 mg l^{-1} IAA (indoleacetic acid). The shoots were then transferred to 10 cm pots containing a 1:1 mixture of barnyard manure and sand. The pots were covered with jars in order to keep the relative humidity high. The shoots began to take hold after fifteen to twenty days. From then on, the jars were removed once a day for few hours, and the plants were sprayed with water. After one month, the jars were removed entirely, and the plants were transferred to the greenhouse and gradually exposed to normal conditions. The survival rate of the plants was recorded thirty days after they were transferred to the pots.

RESULTS

Calli did not form on plain MS medium, but did form on medium supplemented with any of the growth regulators tested (Fig. 1).

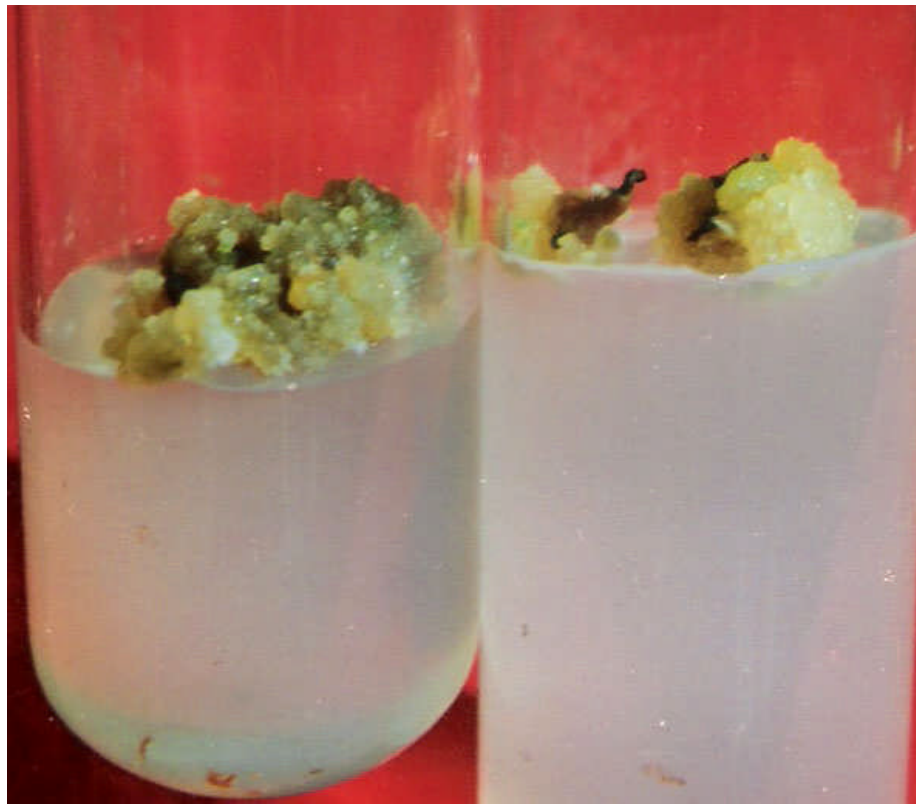

Figure 1. Callus from leaf explant of *Gerbera jamesonii* growing on MS medium supplemented with 1.5 mg l^{-1} 2,4-D

Figure 2 (page 161 →). Photomicrographs illustrating cell growth, cell division and plant regeneration using suspension cultures of callus derived from leaf explants of *Gerbera jamesonii*

A. Two-cell stage.
B. Elongated to kidney-shaped cells.
C. Eight-cell stage.
D. Micro-colony of more than thirty-two cells in liquid MS medium supplemented with 1.5 mg l^{-1} 2,4-D.
E. Formation of micro-calli on solid medium supplemented with 1.5 mg l^{-1} 2,4-D.
F. Formation of macro-calli.
G. Differentiation of calli into adventitious shoots on MS medium. supplemented with 1 mg l^{-1} BA.
H. Hardened plants after two months of growth in pots containing a 1:1 mixture of barnyard manure and sand.

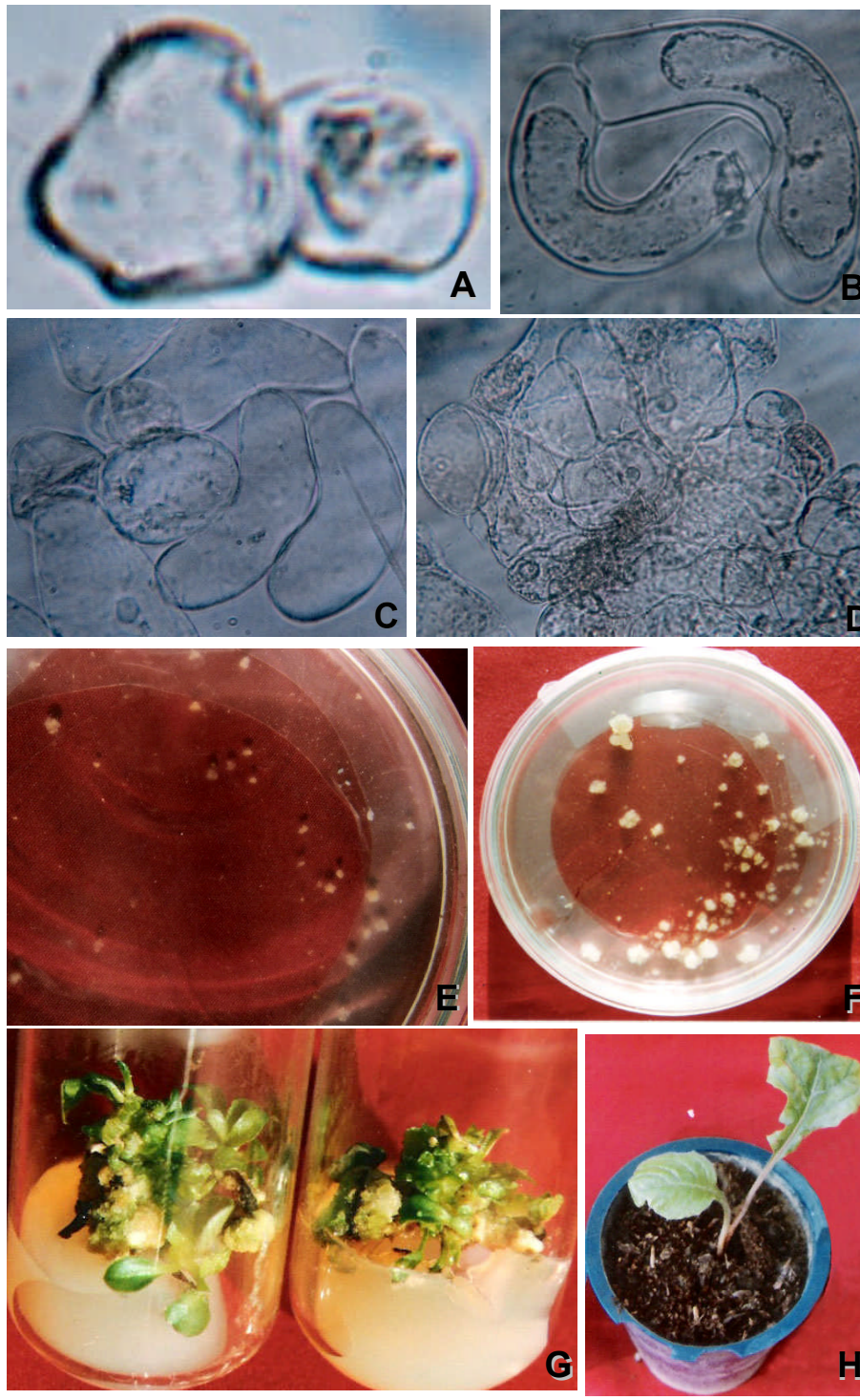
A
B
C
D
E
F
G
H

Table 1. Effect of growth regulators on the percentage of explants producing callus, callus growth and callus type in *Gerbera jamesonii*

Growth regulator [mg l^{-1}]		Percentage of explants producing callus*	Callus growth	Callus type
IBA	1.0	13.7	++	friable
	1.5	20.0	++	friable
	2.0	18.7	++	friable
NAA	1.0	67.0	+++	friable and nodular
	1.5	77.5	+++	friable and nodular
	2.0	79.0	+++	friable and nodular
2,4-D	1.0	93.0	+++	friable
	1.5	95.0	++++	friable
	2.0	93.7	++++	friable
BA	1.0	90.0	+++	compact and nodular
	1.5	91.5	+++	compact and nodular
	2.0	92.0	+++	compact and nodular
	3.0	97.5	+++	compact and nodular
	5.0	89.5	+++	compact and nodular
Kinetin	1.0	28.0	++	friable and nodular
	1.5	25.5	++	friable and nodular
	2.0	28.7	++	friable and nodular
	3.0	24.0	++	friable and nodular
	5.0	27.0	++	friable and nodular
Control		0		
$CD_{0.05}$**		5.9		

Callus growth: ++: good; +++: very good; ++++: excellent
*Mean of thirty explants
**Critical difference at P<0.05

Table 2. Effect of growth regulators on the percentage of calli producing shoots and on the mean number of shoots per callus in *Gerbera jamesonii*

Growth regulator [mg l^{-1}]		Percentage of calli producing shoots*	Mean number of shoots per callus
BA	1.0	68.7	10.0
	1.5	69.0	8.2
	2.0	70.5	8.0
	3.0	75.0	9.0
	5.0	71.0	2.5
Kinetin	1.0	2.0	2.0
	1.5	2.2	1.0
	2.0	1.8	2.0
	3.0	2.5	2.0
	5.0	8.5	2.0
Control		0	0
$CD_{0.05}$**		3.35	1.55

*Mean of thirty explants
**Critical difference at P<0.05

The percentage of explants producing callus was highest with 1.5 mg l^{-1} 2,4-D. Callus growth was best with 2.0 mg l^{-1} 2,4-D. Callus type depended on the growth regulator used, but was not affected by the dosage (Tab. 1).

Calli induced with IBA, NAA and 2,4-D did not regenerate shoots. Calli induced with BA and kinetin produced shoots after six weeks on induction medium. On medium with 3.0 mg l^{-1} BA, 75% of the calli produced shoots. On medium with 5.0 mg l^{-1} kinetin, 8.5% produced shoots. The average number of shoots per callus was about nine with BA, and about two with kinetin (Tab. 2).

After seven days of incubation on the shaker, the cells were isolated from the friable calli. Cell density was increased by subculturing the suspensions once a week into fresh liquid medium. Cell density reached a maximum after eight weeks of incubation.

The cells began to divide within one week of the start of the suspension culture. A septum formed before each cell divided into two daughter cells (Fig. 2A). The dividing cells elongated into a kidney shape (Fig. 2B). These two cells grew before dividing again (Fig. 2C). Within one week of the first cell division, the micro-colonies had reached the eight-cell stage (Fig. 2D).

The cells were allowed to divide in the liquid medium until micro-colonies of more than thirty-two cells had formed. They were then cultured on the same media solidified with either 6 or 8 grams of agar per liter. Within four weeks brown spherical micro-calli formed (Fig. 2E). These were allowed to grow into macro-calli (Fig. 2F). The macro-calli were then grown for three or four weeks on MS medium containing 1.5 mg l^{-1} 2,4-D, after which they were transferred to MS medium supplemented with 1 mg l^{-1} BA to induce the formation of adventitious shoot buds.

The buds were cultured on the same medium, and produced shoots after two or three weeks (Fig. 2G). The shoots were then rooted *in vitro*. After thirty days, between 70 and 80% of the shoots were still alive and had given rise to complete plantlets (Fig. 2H).

DISCUSSION

The callus induction was best with 1.5 and 2.0 mg l^{-1} 2,4-D. In a previous study on *G. jamesonii*, callus growth was best when explants from fully expanded leaves were induced on MS medium containing 1.0 mg l^{-1} of either IBA, NAA, or BA (Parthasarathy et al., 1997). In another study, callus translucency and regeneration were best when capitulum explants were induced on medium containing both 2.0 mg l^{-1} BA and 0.5 mg l^{-1} IAA (Arello et al., 1991).

Gerbera plants have been regenerated from leaves in other studies (Xu et al., 2002; Tyagi and Kothari, 2004; Ray et al., 2005; Kumar and Kanwar, 2006). In the present study, 75% of the calli induced with various concentrations of BA differentiated to yield an average of about nine shoots per

callus. In a previous study, an average of five to eight shoots were formed by calli from *Gerbera* leaf explants that were induced on MS medium containing 4.0 mg l^{-1} kinetin and 0.1 mg l^{-1} IAA (Tyagi and Kothari, 2004). In another study, calli induced with BAP yielded an average of 14.2 shoots for the cultivar 'Dilmaya', and 6.8 shoots for the cultivar 'SWM' (Parthasarathy and Nagaraju, 1999). In another study, calli induced on 2.2 μM BA and 0.3 μM IAA yielded an average of 4.9 shoots for the cultivar 'Mariola', 2.6 shoots for the cultivar 'Rebecca', and 2.4 shoots for the cultivar 'Boy' (Orlikowska et al., 1999). The number of shoots per callus may therefore depend on the cultivar and type of explant used.

Callus from leaf explants grew best on medium containing either 1.5 or 2.0 mg l^{-1} 2,4-D. These calli were friable and incapable of regenerating shoots, but were able to be used to start suspension cultures consisting of single cells and cell aggregates in a moving liquid medium. In another study, the cellular characteristics of liquid cultures of *G. jamesonii* hybrida were examined (Ruffoni and Massabo, 1991). Cell isolation and separation were best when the cultures were grown in the dark on a shaker set at 100 rpm in liquid medium containing 1.5 mg l^{-1} 2,4-D. Cell suspension cultures have also been started by placing friable callus derived from leaf explants of *Oldenlandia affinis* in medium containing 0.4 mg l^{-1} 2,4-D (Seydel and Dornenburg, 2006).

In the present study, the best results were obtained with an inoculum of 0.8 to 1.0 grams of callus per twenty milliliters of liquid medium. Growth was best on liquid MS medium 1.5 mg l^{-1} 2,4-D. These results agree well with those of a previous study, in which 0.3 g of callus from *Tulipa praestans* was grown in 5 ml of liquid medium (Famelaer et al., 2000). The optimum plating cell density was found to be between 10,000 and 30,000 cells per milliliter.

In the present study, cell division and micro-callus formation were better in aggregates of two to five cells than in isolated cells. This agrees well with a previous study on *Shorea roxburghii*, in which small aggregates of five or six cells divided and formed callus, whereas isolated cells did not (Scott et al., 1988). This may be because single cells are more likely to have been damaged during isolation. Or it may be because single cells cannot adapt after being transferred from liquid medium to solid medium. In solid medium, nutrients are depleted and inhibitory metabolites accumulate in the immediate vicinity of the individual cells (Torry and Reinert, 1961).

The regeneration of plants from suspension cultures is an additional method by which *Gerbera* varieties can be improved. The probability of mutants arising in adventitious shoots is very high. This new propagation method promises to be a useful tool in biotechnology studies on *in vitro* cell selection, protoplast fusion and genetic transformation.

REFERENCES

Aswath C., Choudhary M.L. 2002. Rapid plant regeneration from *Gerbera jamesonii* Bolus callus cultures. ACTA BOT. CROATICA 61: 125-134.

Arello E.F., Pasqual J.E., Pinto B.P., Barbosa M.H.P. 1991. *In vitro* establishment of explants and seedling regeneration in *Gerbera jamesonii* Bolus ex Hook by tissue culture. PESQUISA AGROPECUARIAN BRASILEIRA 26: 269-273.

Famelaer I., Ennik E., Creemers M.J., Eikel-Bloom D., Van Tuyl J.M., Cardic A. 2000. Initiation and establishment of long lived callus and suspension cultures of *Tulipa praestans*. ACTA HORT. 508: 247-251.

Gomez K.A., Gomez A.A. 1984. Statistical procedures for agricultural research. John Wiley and Sons, New York.

Hedtrich C.M. 1985. Production of shoots from leaves and propagation of *Gerbera jamesonii*. GARTENBAUWISSENSCHAFT. 44: 1-3.

Kumar S., Kanwar J.K. 2006. Regeneration ability of petiole, leaf and petal explants in gerbera cut flower cultured *in vitro*. FOLIA HORT. 18: 57-64.

Kumar S., Kanwar J.K., Sharma D.R. 2004. *In vitro* regeneration of *Gerbera jamesonii* Bolus from leaf and petiole explants. J. PLANT BIOCHEM. BIOTECHNOL. 13: 73-75.

Laliberte S., Chretien I., Veith J. 1985. *In vitro* plantlet production from young capitulum explants of *Gerbera jamesonii*. HORT. SCI. 20: 137-139.

Modh F.K., Dhaduk B.K., Shah R.R. 2002. Factors affecting micropropagation of gerbera from capitulum explants. J. ORNM. HORT. 5: 4-6.

Murashige T., Sepra M., Jones J.B. 1974. Clonal multiplication of gerbera through tissue culture. HORT. SCI. 9: 175-180.

Murashige T., Skoog F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. PLANT PHYSIOL. 15: 473-497.

Orlikowska T., Nowak E., Marasek A., Kucharska D. 1999. Effects of growth regulators and incubation period on *in vitro* regeneration of adventitious shoots from gerbera petiole. PLANT CELL TISS. ORG. CULT. 59: 95-102.

Parthasarathy V.A., Nagaraju V. 1999. *In vitro* propagation in *Gerbera jamesonii* Bolus. INDIAN J. HORT. 56: 82-85.

Parthasarathy V.A., Parthasarathy U., Nagaraju V., Mishra M. 1997. Callus induction and subsequent plant regeneration from leaf explants of *Gerbera jamesonii.* FOLIA HORT. 9: 83-86.

Pierik R.L.M., Jansen J.L.M., Maasdam A., Binnendijk C.M. 1975. Optimization of gerbera plantlet production from excised capitulum explants. SCI. HORT. 3: 351-357.

Pierik R. L.M., Segers T.A. 1973. *In vitro* culture of midrib explants of gerbera: Adventitious root formation and callus induction. ZEITSCHRIFT PFLANZENPHYSIOL. 69: 204-212.

Prasanth M., Sekar K. 2004. Studies on age of explant on callus induction in gerbera cv. Mammut. SCI. HORT. 9: 207-211.

Ray T., Saha P., Roy S.C. 2005. *In vitro* plant regeneration from young capitulum explants of *Gerbera jamesonii*. PLANT CELL BIOTECH. MOL. BIOL. 6: 35-40.

Reynoird J.P., Chriqui D., Noin M., Brown S., Marie D. 1993. Plant

propagation from *in vitro* leaf culture of several gerbera species. PLANT CELL TISS. ORG. CULT. 33: 203-210.

Ruffoni B., Massabo F. 1991. Tissue culture in *Gerbera jamesonii* hybrida. ACTA HORT. 289: 147-148.

Seydel P., Dornenburg H. 2006. Establishment of *in vitro* plants, cell and tissue cultures from *Oldenlandia affinis* for the production of cyclic peptides. PLANT CELL TISS. ORG. CULT. 85: 247-255.

Scott E.S., Rao A.N., Loh C.S. 1988. Production of plantlets of *Shorea roxburghii* G. Don. from embryonic axes cultured *in vitro*. ANN. BOT. 61: 233-236.

Torry J.G., Reinert J. 1961. Suspension culture of higher plant cells in synthetic medium. PLANT PHYSIOL. 36: 483-491.

Tyagi P., Kothari S.L. 2004. *In vitro* regeneration of *Gerbera jamesonii* (H. Bolus ex Hook f.) from different explants. INDIAN J. BIOTECHNOL. 3: 584-586.

Xi M., Shi J.S. 2003. Tissue culture and rapid propagation of *Gerbera jamesonii*. J. WANJING FOREST. UNI. 27: 33-36.

Xu S.Q., Yang S.H., Wi D., Wan J.M. 2002. *In vitro* micropropagation of gerbera leaf. ACTA HORT. SINICA 29: 493-494.

Zheng X.F., Wang J.H., Li M.Y. 2002. Factors affecting organogenesis in *Gerbera jamesonii* Bolus cultures *in vitro*. J. JIANGSU FOREST. SCI. TECHNOL. 29: 29-31.

REGENERACJA ROŚLIN Z ZAWIESIN KOMÓRKOWYCH *Gerbera jamesonii* Bolus

Surinder Kumar i Jitender Kumar Kanwar

S T R E S Z C Z E N I E

Rośliny *Gerbera jamesonii* regenerowały z kultur zawiesinowych komórek pochodzących z eksplantatów tkanek liści. Indukcja kalusowa była najlepsza na pożywce zawierającej 2,4-D w stężeniach 1,5 i 2,0 mg l^{-1}. Wytwarzanie pędów z kalusa oraz liczba pędów z kalusa były najwyższe na pożywce MS zwierającej 1,0 mg l^{-1} BA. Kultury kalusa na pożywce z dodatkiem 2,4-D w stężeniu 1,5 mg l^{-1} były kruche i o niskim potencjale regeneracyjnym. Taki kalus był wybierany do badań zawiesin komórkowych. Zawiesiny komórek były inicjowane w płynnej pożywce MS zawierającej 1,5 mg l^{-1} 2,4-D. Takie zawiesiny były przeszczepiane raz w tygodniu do otrzymania maksymalnej gęstości komórek i przeznaczone do tworzenia mikrokolonii na pożywce płynnej. Z kolei mikrokolonie przekształcały się w kalus kiedy przenoszono je na pożywkę zestaloną lub częściowo zestaloną. Taki kalus ulegał zróżnicowaniu w pędy przybyszowe na pożywce MS uzupełnionej 1,0 mg l^{-1} BA. 70 do 80% roślin pochodzących z takich pędów były wciąż żywe 30 dni po przeniesieniu do doniczek

Słowa kluczowe: gerbera doniczkowa, pożywka płynna, regulatory wzrostu, pędy przybyszowe