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Regeneration of *Lycium barbarum* L. plants from leaf tissue, callus culture and callus protoplasts

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ABSTRACT

The possibility of plant regeneration from leaf tissue, callus and callus protoplasts of *Lycium barbarum L.* has been studied. Leaf segments were cultured on B5 medium (Gamborg et al. 1968) containing 1.5 mg/l 6-benzylaminopurine and 0.5 mg/l α-naphthaleneacetic acid. Regeneration of shoots was initiated after 30 days of cultivation. Callus was obtained from leaf and internode tissues on MS medium (Murashige and Skoog 1962) containing 0.4 mg/l of 2,4-dichlorophenoxyacetic acid. Subsequently, callus was successfully subcultured on the same medium with 1 mg/l of 2,4-dichlorophenoxyacetic acid and 0.2 mg/l α-naphthaleneacetic acid. Organogenesis in callus culture was obtained in the course of 40 days after transferring to TM-4 (Shahin 1984). Protoplasts were isolated from callus tissue grown *in vitro* using an enzymatic method. Cell colonies, minicallus formation and organogenesis were obtained. Shoots were rooted on Murashige and Skoog medium containing 0.1 mg/l α-naphthaleneacetic acid. Regenerated plants were transferred to soil and were grown to maturity. Regenerated plants carried normal morphological traits.

ABBREVIATIONS

BA, 6-benzylaminopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, α-naphthaleneacetic acid; Zea, zeatin; GA_3, gibberellic acid; MS, Murashige and Skoog medium; B5, Gamborg medium.

INTRODUCTION

Methods of cell, tissue and organ culture have been developed for a good number of *Solanaceae* species, such as *Nicotiana*, *Datura*, *Solanum*, *Lycopersicon* and *Petunia*. Species being used for somatic hybridization and genetic transformation are mainly herbaceous plants. Up to now, such a large genus as *Lycium* has not been used in similar studies. At the same time, together with *Grabowskia* and *Phrodus*, it represents one of the 12 tribes of the *Solanaceae* family (Hunziker 1979). *Lycium barbarum L.*, is a woody bush spread all over Eurasia and North Africa and has a number of useful traits. Often it is cultivated for green hedges and as an ornamental plant. It is also used as a medicinal plant (Niu et al. 1985). It is a melliferous plant, highly resistant to different *Phytophthora* races. *L. barbarum* berries contain vitamin C (Grossgaim 1946) and a high percentage of dry substance. On the whole, this species can serve as a rather good experimental material for obtaining somatic hybrids with *Lycopersicon* as well as for experiments on genetic transformation. We have already demonstrated the possibility of regeneration of *L. barbarum* plants from mesophyll protoplasts (Ratushnyak et al. 1989). In this report we present the results on plant regeneration from leaf, callus, and callus protoplasts.

MATERIALS AND METHODS

Plant Material

Seeds were sterilized using standard methods and germinated *in vitro*. Plants were propagated by cuttings and were grown on hormone-free MS-3 medium (Murashige and Skoog 1962) (Table 1).

Culture Media

Media composition for callus culture, organogenesis from leaves, and rooting of shoots are given in Table 1. Shoot regeneration from callus tissue was obtained on TM-4 medium (Shahin 1984). Plant regeneration from callus protoplasts was achieved using the following media: TM-2 (protoplast culture); TM-3 (minicallus formation) and TM-4 (morphogenesis) (Shahin 1984). The pH of the media was adjusted by 0.1N KOH, then agar (Serva) was added, and autoclaved for 15 min at 125°C. The enzyme mixture and the medium for protoplast culture were filter sterilized.

Callus Formation

Leaf segments (5 to 8 mm wide) and young internodes (5 mm long) were placed on MS-1 medium in 90 mm Petri dishes and cultivated under temperature controlled conditions

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in the dark at 25°C. Callus was induced within a month and then subcultured every 20 days on MS-2 under the same conditions.

Regeneration of Plants from Leaves and Callus Tissue

Leaves were transplanted into plastic Petri dishes on B5-1 medium (Gamborg et al. 1968) and cultivated at 26±1°C, 16-hour photoperiod and illumination of 3000 to 4000 lux. A month later, bud formation and shoot morphogenesis were observed. Shoots from callus tissue were regenerated on TM-4 medium (1 mg/l Zea and 0.2 mg/l GA_3) under the same conditions after 40 days of cultivation. Shoots were rooted on MS-3 medium containing 0.1 mg/l NAA, and subsequently transferred to soil.

Table 1. Composition of the media used, mg/l.

	Medium			
	B5-1	MS-1	MS-2	MS-3
Macronutrients	B5	MS	MS	1/2MS
Micronutrients	B5	MS	MS	MS
Nicotinic acid	1	1	1	–
Thiamine-HCl	10	2	2	1
Pyridoxine-HCl	1	1	1	–
Riboflavine	–	0.2	0.2	–
Adenine sulfate	–	2	2	–
Casein hydr.	300	500	500	–
L-Glutamine	–	20	20	–
Myo-inositol	100	100	100	100
BA	1.5	–	–	–
NAA	0.5	–	0.2	0.1
2,4-D	–	0.4	1	–
Sucrose, g/l	20	20	20	20
Serva agar, g/l	7	7	7	7
pH = 5.7				

Protoplast Isolation and Culture

Approximately 1 g of two-week-old callus tissue was put into 5 ml of an enzyme mixture (pH=5.6), containing 2.2% cellulase "Onozuka R-10", 1.2% pectinase, 0.8% macerozyme R-10 (Serva), 1% driselase (Sigma), 0.8% cellulysine (Calbiochem), 0.5M sucrose and 5 mM $CaCl_2$, and then incubated in glass Petri dishes (ø 60 mm) for 18 hours under temperature-controlled conditions in the dark at 25°C. Protoplasts were filtered through a nylon sieve (ø 100 m) and then centrifuged for 3 min at 100 g. The floating protoplasts were collected and washed twice with W5 salt solution (Medgyesy et al. 1980) for 2 min at 60 g. Protoplast density was measured using a hemocytometer. Protoplasts were cultured in 1.5 ml of TM-2 medium with 0.5 Zea and 1 mg/l NAA, using 40 mm plastic Petri dishes during the first five days in the dark at 25°C, then under 600 lux illumination 16-hour photoperiod and 26±1°C. The plating efficiency was determined in the 2nd to 3rd week of incubation as the ratio between the number of dividing cells and the number of cultivated protoplasts multiplied by 100. Cell colonies cultured on TM-3 agar solidified medium with 0.5 mg/l BA and 0.2 mg/l 2,4-D, formed minicallus which then were transferred to TM-4 regeneration medium supplied 1 mg/l Zea and 0.2 mg/l GA_3. After 50-60 days of culture, the regenerated shoots were rooted on MS-3 medium. Formation of minicallus, morphogenesis and rooting of shoots were successful under the conditions of a 16-hour photoperiod (fluorescent lamps, 3000 to 4000 lux) at 26±1°C. The morphogenetic index was determined as a ratio of the callus capable of regeneration to the total number of callus.

RESULTS

Callus Culture

Several media based on MS mineral elements were tested for callus induction from leaf segments and young internodes. As a result of manipulating the growth substances (Table 2), a suitable variant of MS-1 medium has been found. Callus induced on MS-1 medium was notable for its intensive growth, soft and friable consistence and white colour (Fig. 1). The frequency of callus formation was 96%. A similar search was conducted for a suitable medium for callus subculture. Callus induced on MS-1 medium was grown on different variants of MS medium (Table 3). MS-2 medium turned out to be the most suitable one for long-term culture of callus tissue (Fig. 2). This callus was used as a source for the isolation of protoplasts and regeneration of plants.

Table 2. Callus tissue induction with different combinations of phytohormones.

Hormone composition	Concentration, mg/l	Growth characteristics
2,4-D	0.1–0.8	+ + +
2,4-D;NAA	0.1–0.4;0.05–0.2	+ +
2,4-D;Kin	0.2;0.1–0.3	+ +
2,4-D;BA	0.1–0.3;0.02	+
2,4-D;BA;NAA	0.1–0.2;0.04;0.1	+ +
2,4-D;BA;IAA	0.1;0.02;0.05	+

a) '+ + +'–good; '+ +'– moderate; '+'– poor.

Table 3. Callus growth with different combinations of phytohormones.

Hormone composition	Concentration, mg/l	Growth characteristics
2,4-D	0.4	+ +
2,4-D;NAA	0.1–1;0.05–0.2	+ + +
2,4-D;Kin	0.2;0.3	+
2,4-D;BA	0.1;0.02	+
2,4-D;BA;NAA	0.1–1;0.2;0.05–0.5	+
2,4-D;BA;IAA	0.1; 0.02; 0.05	+

a) '+ + +'– good; '+ +'–moderate; '+'– poor.

Plant Regeneration from Leaves and Callus Tissue

The purpose of this investigation was to study the regeneration potential of leaf explants and of callus cultures of *L. barbarum*. Experiments were carried out using TM-4 regeneration medium. Callus explanted to TM-4 medium became green and compact. By the end of the fifth week of culture, 2 to 5 shoots had regenerated from each callus (Fig. 3).

Whole or cut leaves were placed on B5-1 medium. During the first two weeks of culture the appearance of primary callus

capable of adventitious shoot formation was observed. As a result of intensive organogenesis (Fig. 4), a number of plantlets were obtained from each leaf segment.

Shoots regenerated from the leaf and from callus tissue were grown on MS-3 medium. Rhizogenesis was observed after two weeks of culture (Fig. 5 a, b). The rooted and normally formed regenerants were grown in pots with soil in the greenhouse (Fig. 6). All plants were characterised by normal morphology of leaves and flowers.

Protoplast Culture and Plant Regeneretion

The population of fresh-isolated callus protoplasts (Fig. 7A) was represented by two types: small (with a diameter of 30 to 50 μm), cytoplasm-rich with or without very small vacuoles; and large (ø 50 to 90 μm), poor in cytoplasm, with big vacuoles. After three days of culture, more than 80% of living protoplasts regenerated a cell wall and increased in size (up to a length of 150 μm) on TM-2 liquid nutrient medium. The plating density was $1-3x10^5$ protoplasts/ml. The first mitotic divisions of cells (Fig. 7B) were observed on the 5th to the 6th day. The culture of cells in this state required the addition of fresh TM-2 medium and transfer of the culture to 90 mm plastic Petri dishes. Otherwise, growth of colonies was noticeably retarded. A similar effect was observed when *L. barbarum* mesophyll protoplasts were cultured (Ratushnyak et al. 1989). It can be explained by the possible accumulation of products in the medium, which inhibit considerably the cell division. The callus protoplast plating efficiency reached 5 to 6% which is approximately one half less than that of mesophyll protoplasts. After fifteen days of culture, the compact cell colonies (Fig. 7C) were

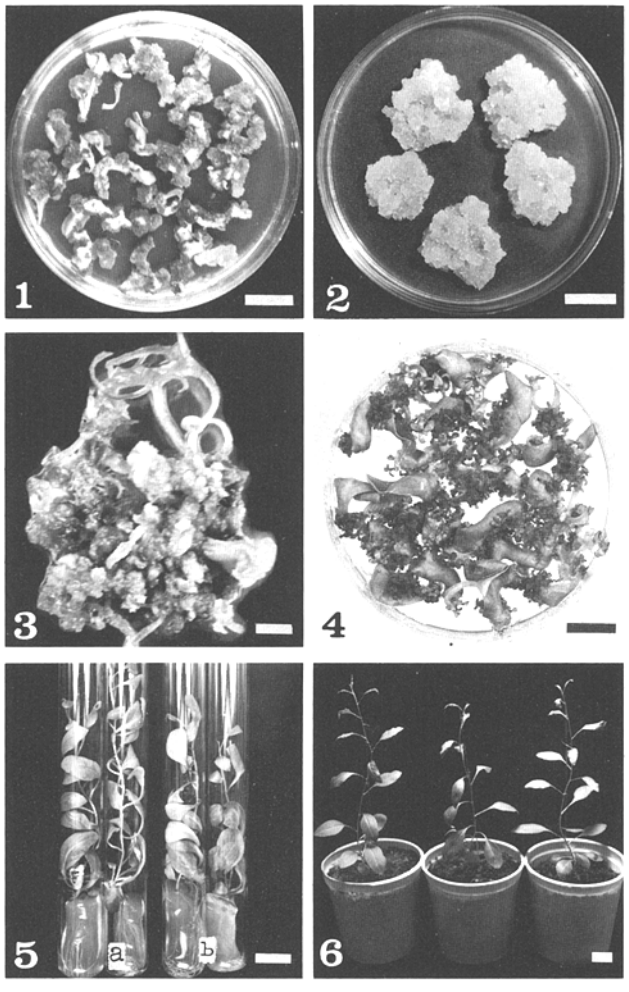

Fig. 1. Callus induction from leaf segments on MS-1 medium (bar=15 mm). Fig. 2. Callus culture on MS-2 medium (bar=15 mm). Fig. 3. Callus morphogenesis on TM-4 medium (bar=4 mm). Fig. 4. Leaf morphogenesis on B5-1 medium (bar=15 mm). Fig. 5. Rooted regenerated plants on MS-3 medium derived from: a) callus; b) leaves (bar=15 mm). Fig. 6. Regenerated plants from callus in soil (bar=20 mm).

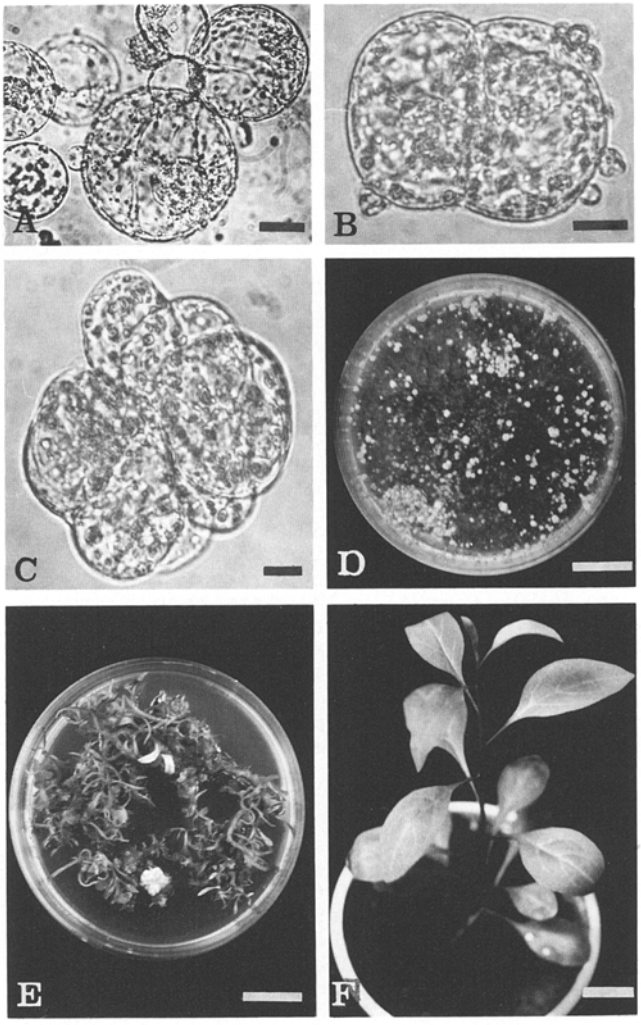

Fig. 7. Plant regeneration from callus protoplasts. A. Freshly isolated callus protoplasts. B. The first division of protoplast. C. Cell colony developed from protoplast. D. Cell colonies on TM-3 medium. E. Organogenesis on TM-4 medium. F. Regenerated plant in soil. Bar represent 26 μm (A), 30 μm (B), 7 μm (C), 19 mm (D, E) and 14 mm (F).

transferred to TM-3 intermediate nutrient medium. White minicallus of 2 to 3 mm (Fig. 7D) was transferred to TM-4 regeneration medium. During the first two weeks of culture the minicallus grew intensively and turned green. Bud formation and regeneration of shoots (Fig. 7E) were very intensive. The morphogenesis index reached 80%. The shoots were separated from the callus and transferred to MS-3 medium. In total, 19 out of the 23 shoots were rooted. The regenerated plants were transferred to soil (Fig. 7F). On the whole, the "callus culture-protoplast-plant" cycle took about three months (Table 4).

Table 4. Stages of regeneration of L. *barbarum* plant from callus protoplasts.

Stages	Media used	Duration of cultivation, days	%
Cell colony formation	TM-2	15	5.5
Minicallus formation	TM-3	8	97
Regeneration of shoots	TM-4	35	80
Rooting of shoots	MS-3	20	82.6

DISCUSSION

Our study has demonstrated the necessity of the presence of 2,4-D in the medium for the process of callogenesis of *L. barbarum*. A relatively small concentration of this auxin (0.4 mg/l) is required for the effective induction of callus from leaves and young internodes. For further growth of the callus 1 mg/l 2,4-D in combination with 0.2 mg/l NAA was required. In this case, the callus morphology and growth rate were quite satisfactory. The proposed technology of obtaining *L. barbarum* callus culture is convenient and efficient enough. The callus obtained is distinguished by high regenerative capacity and provides sufficient numbers of viable protoplasts. The organogenesis was more intensive from leaf explants than from callus. Such a high regenerative potential can be used for genetic transformation *L. barbarum* by the leaf-disk method (Horsch et al. 1985).

A number of reports are aimed at studying *Lycium in vitro* culture. Regenerated plants with different ploidy were obtained from *L. chinense* endosperm callus (Gu et al. 1985). Cultivation of unfertilised ovules of *L. barbarum* led to the same result (Qin et al. 1985). Haploid plants were induced in anther culture of *L. barbarum* and *L. chinense*, and then diploidized (2n=24) (Fan et al. 1982). Callus capable of plant regeneration was induced from hypocotyls, shoots and ovules of *L. barbarum* (Niu et al. 1983). *Citrus* and *Ulmus* callus was used as a source of protoplasts for plant regeneration (Kobayashi et al. 1985; Vardi et al. 1982; Sticklen et al. 1986). Formation of colonies from protoplasts of *L. chinense* was reported by Sun et al. (1982). The present paper demonstrates such a possibility for callus protoplasts of *L. barbarum*, another woody perennial. Undoubtedly, the work on plant regeneration from cells and protoplasts will promote the involvement of *L. barbarum* in the technology of genetic engineering with the purpose of transferring the useful traits of this species to the genome of crop plants.

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