Plant Cell Reports (1999) 18: 797–802

Z. Luthar · B. Bohanec

Induction of direct somatic organogenesis in onion (*Allium cepa* L.) using a two-step flower or ovary culture

Received: 14 October 1998 / Revision received: 19 November 1998 / Accepted: 30 November 1998

Abstract A novel method for direct organogenesis in onion (*Allium cepa* L.) resulting in the formation of multiple shoot structures induced on mature flower buds or ovaries in a two-step culture procedure is described. Flowers were cultured on an induction medium containing 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 2 mg/l 6-benzylaminopurine (BAP). After 6 days (superior to 3 or 12 days), flowers or extracted ovaries were transferred to a differentiation medium containing 2 mg/l thidiazuron (TDZ). Medium solidification with gellan gum was superior to agar or agar/gellan gum mixture. A similar regeneration frequency was achieved at high (100 g/l) and lower (50 g/l) sucrose content. Regeneration was obtained from all 12 cultivars or inbred lines examined, although the efficiency and the occurrence of hyperhydricity varied depending on genotype and procedure used. Studies of plant growth regulators revealed that in the induction medium, the auxin 2,4-D was superior to 5 mg/l naphthaleneacetic acid or picloram, which partially or completely inhibited regeneration. Omitting cytokinin in the induction medium or substitution of BAP with 2 mg/l 2iP lowered regeneration, while substitution with 1 mg/l TDZ was equally effective. In the differentiation medium, lower concentrations of TDZ (1 and 0.5 mg/l) or substitution of TDZ with 5 mg/l BAP were equally or less effective.

Key words Direct somatic organogenesis · Multiple shoot formation · *Allium cepa*

Abbreviations *BAP* 6-Benzylaminopurine · *2,4-D* 2,4-Dichlorophenoxyacetic acid · *NAA* Naphthaleneacetic acid · *TDZ* Thidiazuron

Communicated by H. Lörz

Z. Luthar · B. Bohanec (✉)
Biotechnical Faculty,
Centre for Plant Biotechnology and Breeding,
Jamnikarjeva 101, 1000 Ljubljana, Slovenia
Fax: +386-61-1231088
e-mail: borut.bohanec@uni-lj.si

Introduction

Onion is the second most important vegetable species worldwide and is produced in almost all climatic regions. There has been considerable interest in developing somatic regeneration systems for in vitro multiplication of specific genotypes such as male sterile inbred lines, and in the development of protocols offering the high regeneration ability needed for use in genetic transformation studies. Different organogenic responses have been studied in several onion in vitro culture systems. In general, two different tissues have been used for induction of shoot cultures: (1) inoculation of scale bases excised from the basal parts of bulbs or onion sets (Hussey 1978; Fujieda et al. 1979; Hussey and Falavigna 1980; Kahane et al. 1992) and (2) inoculation of different flower parts such as receptacles (Matsubara and Hihara 1978) or immature flower buds (Pike and Yoo 1990; Mohamed-Yassen et al. 1993). Callus tissues have been induced on a wider range of explant tissues, including bulb, set or seedling radicle (Dunstan and Short 1978), seedling leaf sheets, immature sexual embryos, immature unfertilized ovules, and mature basal plates (Phillips and Luteyn 1983). The most frequently studied growth hormones for shoot induction have been naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP), while for callus induction, picloram, NAA and 2,4-dichlorophenoxyacetic acid (2,4-D) have been used. Similar responses have also been studied in other *Allium* species such as leek (Novak and Havel 1981; Doré 1988; Rauber and Grunewaldt 1988; Silvertand et al. 1996) or Chinese chive (Yasushi and Adachi 1996). However, in vitro responses of other *Allium* species differ from onion in several aspects.

A characteristic of published protocols is a relatively low number of shoot formations per donor plant, protocols based on bulb scale parts resulting in up to 10 shoots per explant, while the explant number per bulb is limited (Fujieda et al. 1979). Protocols based on immature flower buds result in about 10% induced flowers, which produce about 5 shoots per flower (Pike and Yoo 1990) or 42.4 shoots

per umbel (Mohamed-Yassen et al. 1993). In-vitro-grown shoots can be subcultured according to Kahane et al. (1992), but the multiplication cycle requires 3–4 months. On the other hand, the protocol published for somatic embryogenesis (Phillips and Luteyn 1983) requires the induction of callus, and embryogenic regeneration from callus has been demonstrated only for a limited number of genotypes.

The absence of an efficient direct regeneration protocol is probably the reason why onion is one of the last important agricultural plants for which no report of stable genetic transformation has been published. Our studies have focused on two-stage induction – a differentiation procedure starting from mature flowers and ovaries, for which the choice of growth regulators, gelling agents, sucrose content and genotypic responses have been examined. The aim of these studies has been to develop a reliable protocol enabling high-frequency induction of multiple shoot structures via direct organogenesis in a wide range of genotypes.

Materials and methods

The plant material originated from different sources: cultivars were received from genebanks or were purchased at retail, and inbred lines came from the US public breeding program (Dr. M. J. Havey, USDA, Madison, Wisc.). Flower buds at a mature stage but before dehiscence were taken from greenhouse-grown plants and surface sterilized with 16.6 g/l dichloroisocyanuric acid Na_2 salt with the addition of a few drops of Tween 20 for 10 min and washed three times in sterilized water. In procedure 1, flower buds were cultured in 100-mm petri dishes (30 per dish) on induction media for 3, 6, or 12 days. After the induction period, flowers were subcultured on petri dishes containing differentiation media. Procedure 2 differed from procedure 1 in that the flowers were cultured on induction media, and after 6 days (where not said otherwise), ovaries were extracted and cultured on differentiation media. Petri dishes were sealed with Parafilm and exposed to a 16/8 photoperiod at 21–23 °C and illumination of 80 μmol m^{-2} s^{-1}. The basal medium consisted of BDS macro- and microelements and vitamins (Dunstan and Short 1977), 500 mg/l inositol, 200 mg/l proline, pH 6.0; other media components are listed in Table 1.

One-way ANOVA followed by Duncan's multiple-range test was conducted to evaluate differences among treatments. Only experiments performed in the same time interval were compared, and treatments on media I1/D1 or I4/D4 were used as control.

Table 1 Media composition for induction (*I*) and differentiation (*D*)

	Sucrose (g/l)	2,4-D (mg/l)	NAA (mg/l)	Picloram (mg/l)	TDZ (mg/l)	BAP (mg/l)	2iP (mg/l)	Agar (g/l)	Gellan gum (g/l)
I1	100	2				2			2
I2	100	2				2		3.5	1
I3	100	2				2		7	
I4	50	2				2			2
I5	25	2				2			2
I6	50			1		2			2
I7	50			2		2			2
I8	100		5			2			2
I9	50	2							2
I10	50	2			1				2
I11	50	2					2		2
D1	100				2				2
D2	100				2			3.5	1
D3	100				2			7	
D4	50				2				2
D5	25				2				2
D6	50				1				2
D7	50				0.5				2
D8	100					5			2
D9	50					5			2

Results

Flowers and ovaries cultured on induction media and transferred to differentiation media formed the first visual structures after 3 weeks in culture. At this stage, regenerated structures had a globular embryogenic appearance and were completely white (Fig. 1A). The first visual shoot organogenesis appeared on globular structures in the following week (Fig. 1B). Part of the shoot structure elongated in the next 2 weeks on the differentiation medium, individual shoots being approximately 2 cm long (Fig. 1C–E). Such shoots were divided and subcultured on the elongation media, on which they elongated and produced normal plantlets (Fig. 1F). A proportion of these organogenic structures remained as nodular bumps. When such clusters were subcultured on hormone-free media, elongation of shoots occurred; however, the organogenic potential was preserved for at least three subcultures. The leaves remained white almost until the end of growth on the differentiation media (Fig. 1D, E) and became green in the next subculture (Fig. 1F). Results were scored as the number of flowers or ovaries producing organogenic multiple-shoot structures. The number of individual shoots per flower or ovary is difficult to determine because smaller nodular structures are present in addition to elongated shoots. The average cluster at the end of subculture was composed of five to ten elongated shoots and the remaining compact organogenic tissue. Shoots are normal and can be elongated, rooted, and acclimatized in the same way as other micropropagated onion shoots.

Effect of gelling agents

Media solidified using agar (I1/D1), agar/gellan gum mixture (I2/D2) or gellan gum (I3/D3) were tested using procedures 1 and 2 (Table 2). The highest shoot regeneration occurred on media solidified with gellan gum in both procedures (42.0% for flowers and 35.9% for ovaries) but more shoots exhibited a hyperhydric appearance on gellan-gum-solidified media than on agar or agar/gellan gum mixture.

Duration of induction stage

Establishment of an optimal duration of induction was studied using procedure 2 on I1/D1 media. The duration of induction (3, 6, or 12 days) had a significant effect on

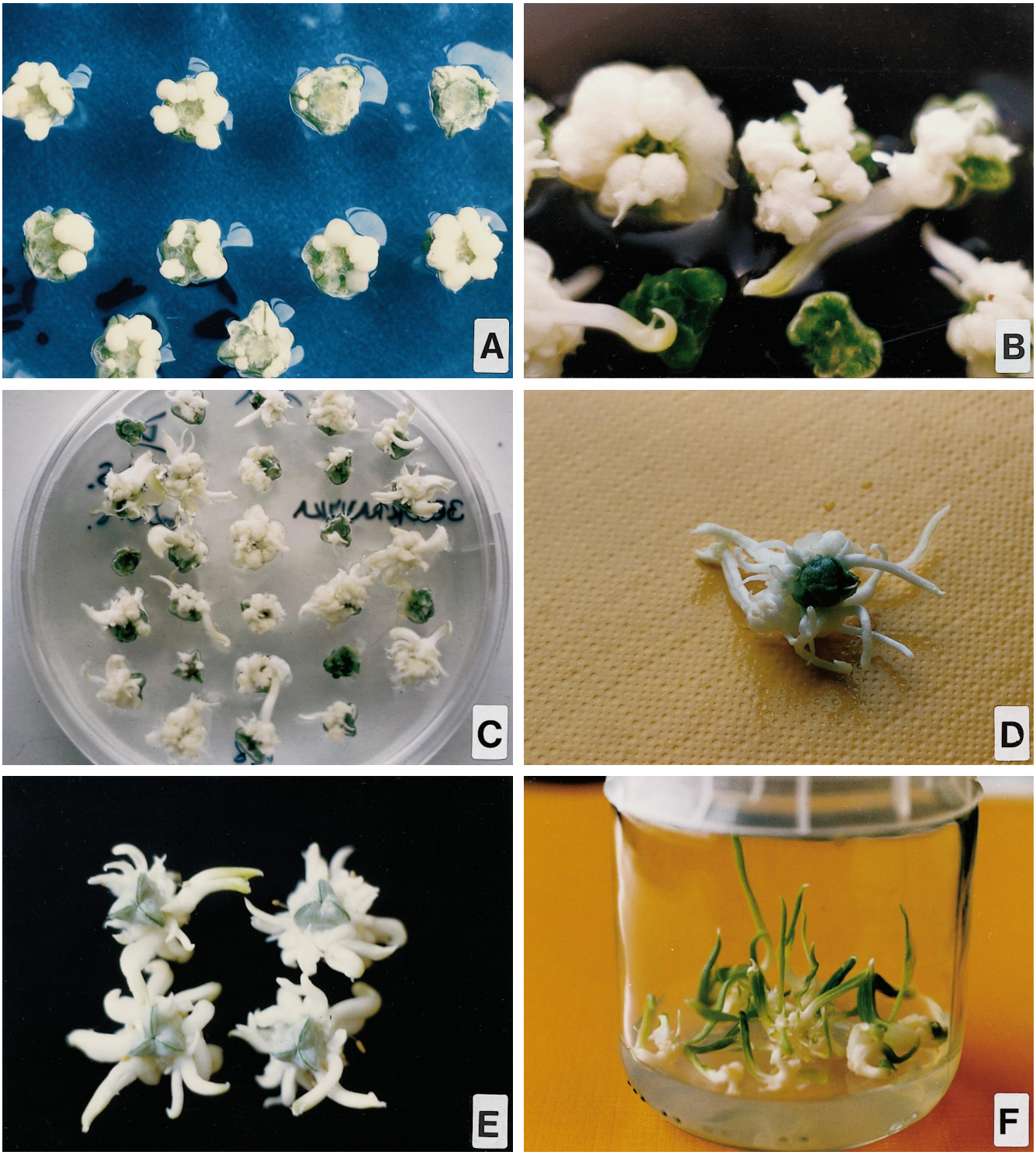

Fig. 1 A–F Formation of multiple shoot structures on ovaries or flowers on gellan-gum-solidified differentiation medium (D1) containing 2 mg/l TDZ. **A** Initiation of globular structures on the bases of ovaries. **B**, **C** Two stages of multiple shoot proliferation on ovaries. **D** Multiple shoots formed on an ovary at the subculturing (subdivision) stage. **E** Multiple shoots formed on flowers. **F** Multiple shoots on hormone-free elongation medium

Table 2 Induction of multiple-shoot formation and observation of hyperhydration on flowers and ovaries extracted from flowers on media supplemented with agar, agar/gellan gum mixture or gellan gum. Percentages followed by identical letters are not significantly different according to Duncan's multiple-range test ($P<0.05$)

Medium	Flowers		Ovaries		
	Flowers plated (*n*)	Organogenic flowers (%)	Ovaries extracted (*n*)	Organogenic ovaries (%)	Hyperhydration (%)
I1/D1	419	42.0 b	797	35.9 c	20.7
I2/D2	420	24.3 ab	814	24.0 b	3.7
I3/D3	388	15.7 a	872	10.7 a	1.4

Table 3 Induction of multiple-shoot formation and observation of hyperhydration on ovaries extracted from flowers cultured on induction medium for 3, 6, or 12 days. Percentages followed by identical letters are not significantly different according to Duncan's multiple-range test ($P<0.05$)

Medium	Duration of induction (days)	Ovaries extracted (*n*)	Organogenic ovaries (%)	Hyperhydration (%)
I1/D1	3	300	43.0 b	41.1
I1/D1	6	300	61.0 c	29.78
I1/D1	12	319	17.6 a	62.5

regeneration (Table 3). The highest shoot regeneration (61.0%) occurred on ovaries following 6 days induction; shorter or longer induction resulted in lower regeneration – 43.0% and 17.6% respectively – and more hyperhydration.

Effect of sucrose concentration

Concentrations of sucrose in the induction and differentiation media were studied using two genotypes and procedure 2 (Table 4). With two genotypes, media supplemented with 50 g/l sucrose (I4/D4) were superior to 100 g/l (I1/D1) and 25 g/l (I5/D5), but for only one genotype were these differences statistically significant. Hyperhydration was lowest in both genotypes at the intermediate sucrose concentration.

Effect of auxin and cytokinin content in induction media

The effects of three auxins (2,4-D, NAA, and picloram) and three cytokinins (TDZ, BAP and 2iP) were studied using procedure 2 (Table 4). Substitution of 2,4-D with NAA in the induction media (I8) gave negative results with two genotypes, while substitution by picloram at two tested concentrations (I6, I7) resulted in a significantly lower induction frequency and higher hyperhydration with both tested genotypes.

The absence of cytokinin in the induction media (I9) resulted in a significantly lower induction frequency with both tested genotypes compared to the control (I4). Substitution of TDZ with 2iP (I11) decreased regeneration with both genotypes, while a lower concentration of TDZ (I10) slightly improved regeneration with one genotype and reduced it in the other, neither difference being statistically significant.

Effect of cytokinin content in differentiation media

The results of altering cytokinin levels are presented in Table 4. Comparison of standard differentiation medium

Table 4 Induction of multiple-shoot formation and observation of hyperhydration on ovaries extracted from flowers cultured on media with different sucrose content or various auxin and cytokinin concentrations in induction and differentiation media. Percentages (within experiments indicated by table division) followed by identical letters are not significantly different according to Duncan's multiple-range test ($P<0.05$)

Medium	Genotype	Ovaries extracted (*n*)	Organogenic ovaries (%)	Hyperhydration (%)
Effect of sucrose concentration				
I1/D1	Belokranjka	539	21.3 a	13.9
I4/D4	Belokranjka	538	26.2 a	3.5
I5/D5	Belokranjka	541	25.7 a	5.0
I1/D1	MSU5718B	446	6.7 a	25.0
I4/D4	MSU5718B	451	17.7 b	11.2
I5/D5	MSU5718B	440	11.1 a	24.5
Effect of auxin and cytokinin content in induction media				
I8/D1	MSU8155B	316	0	0
I8/D1	B2923B	333	0	0
I4/D4	Belokranjka	845	35.6 b	3.6
I6/D4	Belokranjka	540	11.7 a	13.5
I7/D4	Belokranjka	538	14.9 a	23.7
I4/D4	MSU5718B	362	26.2 b	11.0
I6/D4	MSU5718B	504	6.7 a	16.2
I7/D4	MSU5718B	476	6.9 a	27.3
I4/D4	Belokranjka	845	35.6 c	3.6
I9/D4	Belokranjka	540	5.6 a	8.4
I10/D4	Belokranjka	532	43.2 c	0.4
I11/D4	Belokranjka	538	16.5 b	4.5
I4/D4	MSU5718B	362	26.2 c	11.0
I9/D4	MSU5718B	484	8.5 a	12.2
I10/D4	MSU5718B	482	19.3 bc	12.4
I11/D4	MSU5718B	482	12.2 ab	27.1
Effect of cytokinin content in differentiation media				
I1/D1	Belokranjka	718	39.4 b	8.8
I1/D8	Belokranjka	330	4.5 a	36.7
I4/D4	Belokranjka	845	35.6 c	3.6
I4/D7	Belokranjka	537	17.3 a	9.7
I4/D6	Belokranjka	541	27.0 b	2.7
I4/D4	MSU5718B	362	26.2 a	11.0
I4/D7	MSU5718B	425	26.4 a	9.8
I4/D6	MSU5718B	423	21.0 a	9.0
I1/D1	B2923B	164	12.8 a	28.6
I4/D4	B2923B	167	43.1 b	22.3
I1/D8	B2923B	166	18.7 a	32.3
I4/D9	B2923B	166	27.1 a	20.0

Table 5 Induction of multiple-shoot formation and observation of hyperhydration, callus formation and indirect regeneration on flowers and ovaries extracted from flowers of 12 different cultivars or inbred lines. Percentages followed by identical letters are not significantly different according to Duncan's multiple-range test ($P < 0.05$)

Genotype	Flowers					Ovaries		
	Flowers plated (*n*)	Organogenic flowers (%)	Flowers producing basal callus (%)	Shoots formed on callus (%)	Hyperhydration (%)	Ovaries extracted (*n*)	Organogenic ovaries (%)	Hyperhydration (%)
Belokranjka	712	45.4 ef	16.3	6.5	2.3	718	39.4 d	8.8
Stuttgarter Riesen	897	20.1 abc	18.6	6.7	5.2	947	14.6 ab	6.1
Timor	399	10.8 ab	13.5	5.3	5.5	319	29.2 bcd	30.1
Shenshu Yellow	326	17.2 abc	22.4	5.5	10.1	419	25.8 bcd	15.7
Yamaguchi Koudaka	577	31.2 cde	9.5	2.8	0.5	584	28.8 bcd	14.6
XPH 3371 F_1 (Asgrow)	368	25.5 bcd	13.3	7.9	2.0	357	15.9 abc	18.4
Texas Early Grano 502	309	15.9 abc	27.5	9.1	5.2	318	32.1 cd	3.4
Inb. Line B2355B	477	19.5 abc	26.6	9.2	3.3	467	37.0 d	41.3
Inb. Line B2923B	1139	39.2 def	24.1	5.4	20.8	1339	14.9 abc	22.4
Inb. Line MSU2935B	446	57.2 f	8.8	2.5	13.2	627	25.5 bcd	21.6
Inb. Line MSU5718B	529	44.0 def	24.6	4.9	25.3	616	57.9 e	13.2
Inb. Line MSU8155B	748	3.6 a	2.5	1.1	10.0	955	4.1 a	23.1

containing 2 mg/l TDZ (D1, D4) with a medium containing 5 mg/l BAP (D8, D9) revealed that substitution of TDZ with BAP decreased regeneration in cv. Belokranjka (at 100 g/l sucrose) and in line B2923B (at 50 g/l sucrose), while other differences were not statistically significant. Lowering the TDZ concentration to 1 and 0.5 mg/l (D6, D7) decreased regeneration in cv. Belokranjka but gave almost equal results in line MSU5718B.

Genotype effect

An examination of the influence of genotype revealed differences among genotypes depending on the procedure used (Table 5). When flowers were cultured on induction and differentiation media (procedure 1), callus developed on the bases of some flowers. Occasionally, adventitious shoot regeneration occurred on such calli, and these were scored separately from those produced via direct organogenesis. When ovaries were extracted (procedure 2), no callus was formed.

All tested varieties produced multiple organogenic structures, although induction percentages differed and differences among cultivars were statistically significant in both procedures. The highest regeneration rates were 57.9% (procedure 2) and 45.4% (procedure 1). Line MSU8155B exhibited the lowest regeneration rate with both procedures, and it flowered 3 weeks later than the others, so in addition to genotype, an environmental effect (higher temperature in greenhouse) could also have had an impact on the lower regeneration. One-half of the varieties responded with similar induction frequencies in both procedures, while others differed, some responding better to procedure 1, some to procedure 2. The appearance of hyperhydric shoots also differed among cultivars, and was lower on flowers (procedure 1) than on ovaries (procedure 2).

Discussion

Our experiments have demonstrated that mature onion flowers or ovaries could be induced to produce multiple organogenic structures via direct regeneration. The induction procedure and media components are very similar to those used for gynogenic embryo induction in onion (Bohanec et al. 1995; Jakše et al. 1996), the major differences between the protocol used for gynogenesis and this procedure (protocol 2) being the use of BDS instead of B5 medium for induction, elevated inositol and vitamin contents, gellan gum instead of agar to solidify the medium, and a shorter induction treatment. These relatively small differences caused a completely different regeneration response from the same onion organs.

Shoot regeneration was higher on gellan-gum-solidified media than with agar or an agar/gellan gum mixture, but more shoots exhibited a hyperhydric appearance. When these results are compared to data obtained in a genotype examination experiment, it became clear that the occurrence of hyperhydricity on gellan-gum-solidified medium was highly dependent on the variety tested. The optimal medium solidifier should be determined for each variety. For those exhibiting high hyperhydration, an agar/gellan gum mixture could be used as an alternative to gellan gum alone.

Duration of the induction treatment had a significant effect on regeneration, 6 days being optimal, while shorter (3 days) and especially longer (12 days) induction treatments inhibited shoot formation. The inhibition caused by prolonged induction may have been a consequence of the relatively high 2,4-D content in the induction media.

Alterations of auxin and cytokinin content in the induction media showed that both growth regulators are needed for optimal initiation. Surprisingly, NAA and picloram, which have been reported as having beneficial effects on

callus induction or differentiation (Hansen et al. 1995; Roy 1995), were completely (NAA) or partially (picloram) ineffective for initiation of direct somatic organogenesis. In the second medium, cytokinins are needed, while the choice and concentration may be genotype dependant.

Testing of 12 different onion varieties or inbred lines primarily showed that multiple shoot structures can be obtained on all tested genotypes using both induction procedures. This result is of special importance for the further use of this protocol, since a low-genotype-specific protocol is needed for plant breeding purposes.

Major differences between the two tested procedures were in the formation of callus on some flower bases in procedure 1, while the average induction frequencies did not differ substantially. We believe that both procedures are useful for micropropagation, although the complete absence of callus formation (reducing the possibility of somaclonal variation) favors the ovary extraction procedure. The total number of induced shoots per donor plant can be very high, assuming that 200–600 flowers are present in each umbel and one to five umbels develop on a single donor plant.

Induction of organogenic structures, especially those formed by the ovary culture procedure, has high experimental potential, in particular for use in transformation attempts, since organogenic structures formed on ovaries, visible at an early globular stage, could be ideal targets for a biolistic or *Agrobacterium* transformation approach.

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