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Improvement of the tissue culture response of seed-derived callus cultures of *Poa pratensis* L.: Effect of gelling agent and abscisic acid

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Abstract

The effects of gelling agent and abscisic acid on the morphogenetic response of seed-derived callus cultures of *Poa pratensis* L. were investigated. On medium solidified with Gelrite, the regeneration frequency of the calluses was twice as high as compared to agar-solidified medium. The average number of green shoots per regenerating callus was not influenced by the type of gelling agent used. When abscisic acid was added to the differentiation medium only, or to both the differentiation medium and the regeneration medium, the percentage of calluses with somatic embryos or embryo-like structures increased (up to 29.6%) as compared to the control (16.4%). The plant formation frequency, however, was not affected by abscisic acid.

Introduction

Since *Poa pratensis* is a highly apomictic species (Kordyum 1976), breeding by sexual crossing is difficult. Another way to introduce genetic variation might be through somaclonal variation induced by in vitro culture techniques. To explore this possibility, an efficient in vitro culture system for plant regeneration is a prerequisite. Although monocotyledonous species have been shown to be recalcitrant in in vitro culture, plants can be regenerated from callus cultures, cell suspensions and protoplasts from a number of species (Vasil 1987). A number of papers have been published that describe systems for callus culture and plant regeneration in *P. pratensis* using, as explant material, mature zygotic embryos (Boyd & Dale 1986; McDonnell & Conger 1984) or immature inflorescences and seeds (Van der Valk et al. 1989). Regeneration frequencies were low when using zygotic embryos and seeds. Immature inflorescences are suitable explant material, but they are only available for a short period when the plants start to flower. Seeds are available year-round and in large quantities, which makes it possible to perform large, reproducible experiments, but the regeneration frequency of seed-derived callus cultures is low (up to 3%, Van der Valk et al. 1989). Hence it was important to optimise the culture conditions in order to increase the regeneration frequency of this callus.

The type of gelling agent has been reported to influence callus growth and plant regeneration in several species. Agarose was found to be superior to agar in protoplast culture for a number of species (Dons & Colijn-Hooymans 1989). Corn starch increased growth and regeneration in tobacco cell cultures (Henderson & Kinnersley 1988) and barley anther culture (Sorvari 1986). The use of Gelrite promoted regeneration of protoplasts of red cabbage (Koda et al. 1988)

and callus initiation on seed hypocotyls of cotton (Zimmerman & Robacker 1988). In banana callus cultures, discoloration of tissue and medium was prevented by Gelrite (Huang & Chi 1988).

Regeneration of *P. pratensis* was found to occur predominantly via organogenesis (Van der Valk et al. 1989), but regeneration via somatic embryogenesis is preferred because of the supposed single cell origin of somatic embryos (Vasil & Vasil 1981). Abscisic acid (ABA) has been reported to stimulate somatic embryogenesis in caraway (Ammirato 1984), *Picea glauca* (Dunstan et al. 1988), *Daucus carota* (Kamada & Harada 1981), wheat (Qureshi et al. 1989) and maize (Close & Ludeman 1987).

In this study, the effect of two gelling agents (Gelrite and agar) and the influence of ABA on the morphogenetic response of seed derived callus cultures of *P. pratensis* is described.

Materials and methods

Callus induction

Seeds of *Poa pratensis* 'Geronimo' were used as explant material in all experiments. The seeds were desinfested in two steps. The first treatment was undiluted with commercial bleach solution (10% (w/v) NaOCl) for one h, followed by at least five washes (10 min each) and storage in sterile tapwater at 4°C for two days. In the second step the seeds were treated a second time (10 min) with commercial bleach and rinsed five times with sterile tapwater.

All media used were based on Murashige & Skoog's medium (1962) supplemented with 0.4 mg l^{-1} thiamine HCl, 3% (w/v) sucrose and different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) (Table 1). All media were autoclaved (120°C, 15′). The pH of the media was set at 5.8 before autoclaving.

Callus was cultured following the procedure as described by Van der Valk et al. (1989, Table 1). For callus induction, 30 seeds were sown per Petri dish (9 cm, Greiner) containing 25 ml of callus induction medium. After six weeks of culture, seeds producing compact and/or friable callus were transferred to fresh medium for further growth (17–19 calluses per dish). The calluses were cultured for three weeks before transferring all the calluses to differentiation medium with reduced 2,4-D concentration (0.2 mg l^{-1}, 17–19 calluses per dish). After a further three weeks of culture the calluses were transferred to regeneration medium without 2,4-D (9 calluses per dish).

In all experiments the morphogenetic response was determined after eight weeks of culture on regeneration medium as the percentage of calluses that formed shoots, and the number of green and/or albino shoots per regenerating callus. Each experiment was performed at least two times.

Gelling agent

When comparing the effect of gelling agent on morphogenetic response, all media used (Table 1) were either solidified with agar (Daishin, Brunschwig Chemie, Amsterdam; 0.8% (w/v)) or with Gelrite (Kelco, San Diego; 0.3% (w/v)). The experiment was performed in three independent replications. The second and third replication were also used to determine callus growth. At each subculture and for both gelling agents separately, samples of 50 calluses were taken by selecting every 13th callus on a dish. These calluses were used to measure fresh weight (FW) and dry weight (DW, 16 h at 60°C). During the regeneration phase the dishes were either sealed with Parafilm to allow gas exchange or cling film to limit gas exchange. After eight weeks on regeneration medium, the morpho-

Table 1. Procedure for seed callus induction and regeneration for *P. pratensis* on MS-medium (D = Dark, L = Light).

Callus culture phase	Concentration 2,4-D (mg l^{-1})	Time (weeks)	Culture Conditions
Induction	2	6	D, 25°C
Growth	2	3	D, 25°C
Differentiation	0.2	3	D, 25°C
Regeneration	0	8	16hL/8hD, 25°C

genetic response was determined as described above.

Abscisic acid

All media used in these experiments were solidified with Gelrite. The ABA solution was added after filter sterilisation. The experiment was performed in two independent replications. Callus initiation and culture protocols were identical to the standard procedure (Table 1). The callus differentiation medium was supplemented with ABA at three concentrations. After three weeks of culture the calluses were transferred to regeneration medium without ABA, or to regeneration medium with the same ABA concentration as the differentiation medium (Table 3). Apart from morphogenetic response, the calluses were screened for the formation of roots and somatic embryos or embryo-like structures.

Statistical analysis

Differences between numbers of green and albino shoots were tested using ANOVAs, differences between percentages were tested using logistic regressions (McCullagh & Nelder 1989). The analyses were performed using GENSTAT (Payne et al. 1987).

Results and discussion

Gelling agents

Both on Gelrite and agar-solidified medium, 75% of the seeds produced compact and/or friable callus. This means that no effect of the gelling agent was found on the induction frequency of compact and/or friable callus. This result is in contrast to results obtained in cotton where seed hypocotyls cultured on Gelrite solidified medium produced more calluses (Zimmerman & Robacker 1988).

Figure 1 shows that after six weeks of culture, the fresh and dry weight of calluses grown on Gelrite- solidified medium were twice as high as for the calluses grown on agar-medium. At the next two subcultures (after 9 and 12 weeks), these weight differences were maintained. Taken together, these data show that callus initiation was faster on Gelrite-solidified medium than on agar.

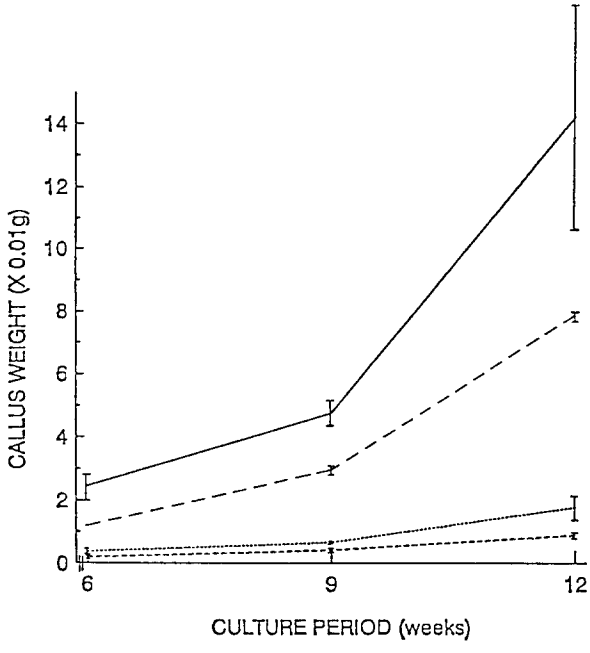

Fig. 1. Comparison of fresh and dry weight (FW and DW) of seed derived calluses cultured on gelrite (GR) and (AG) solidified media. Each point represents the mean of two experiments. 1 SE is indicated. (GR-FW: ————; GR-DW: --------; AG-FW: ------; AG-DW: ------).

The effect of gelling agent and sealing method on morphogenetic response is given in Table 2. The use of Gelrite resulted in a 2 to 2.5 times higher regeneration frequency as compared to agar. The average number of green and albino shoots per regenerating callus was similar for both gelling agents. Some calluses regenerated a high number of shoots, while most calluses regenerated 1 to 4 green shoots (Fig. 2). Sealing the dishes with cling film resulted in a slightly lower regeneration frequency as compared to Parafilm, but this difference was not significant.

The reason for the positive effect of Gelrite on regeneration is not known. It has been suggested that Gelrite contains less growth inhibiting substances than agar (Koda et al. 1988). This hypothesis is supported by the observation that in banana callus cultures discoloration of calluses is prevented by Gelrite but not by agar (Huang & Chi 1988).

Table 2. The influence of the use of different gelling agents and different sealing methods on shoot regeneration of seed derived callus cultures.

Treatment[a]		Number of calluses used	Calluses with shoots (%)	Number of shoots per regenerating callus	
				Green	Albino
AG	PF	1496	5.6a[b]	3.9a	0.2a
	CF	1519	5.1a	3.6a	0.2a
GR	PF	1486	13.8b	4.1a	0.3a
	CF	1538	10.7b	4.1a	0.2a

[a] Agar = AG; Gelrite = GR; Parafilm = PF; Cling film = CF
[b] Means with the same letter are not significantly different at $p = 0.05$.

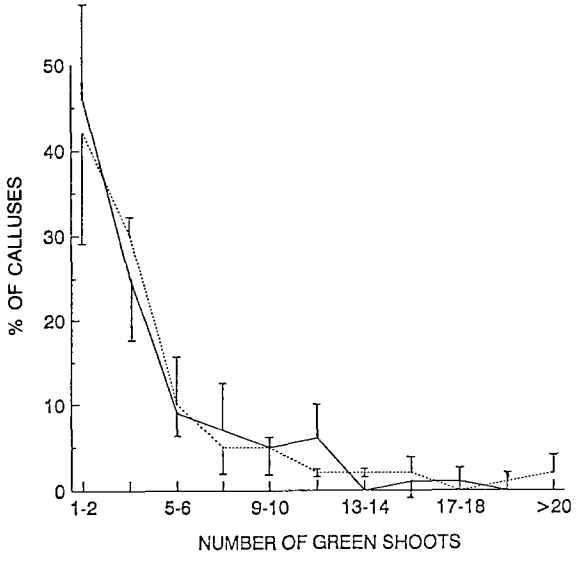

Fig. 2. The average number of green shoots per regenerating callus for calluses cultured on agar and gelrite-solidified media. Each point represents the mean of three experiments. 1 SE is indicated. (Agar: ————; Gelrite ------).

Abscisic acid

The effects of the different ABA treatments are summarised in Table 3. A higher number of calluses produced somatic embryos or embryo-like structures when ABA was present at a concentration of 10^{-6} M in the differentiation medium and of 10^{-7} as well as 10^{-6} M ABA in both the differentiation medium and the regeneration medium. At an ABA concentration of 10^{-5} M, however, no significant increase in the number of calluses forming somatic embryos was found compared to the control. The positive effect of ABA on the formation of somatic embryos was also found for caraway (Ammirato 1984) and *Picea glauca* (Dunstan et al. 1988).

Supplementation of the differentiation and regeneration media with ABA failed to increase the number of shoot-forming calluses. The average number of green shoots per regenerating callus decreased with increasing ABA concen-

Table 3: The effect of different abscisic acid (ABA) treatments on callus development and regeneration.

ABA conc. (M) in		Percentage of calluses with			Number of shoots per callus	
DM[a]	RM[b]	Roots	Somatic embryos	Shoots	Green	Albino
0	0	92.7b[c]	16.4c	9.0a	4.9a	0.3ab
10^{-7}	0	91.4b	17.4bc	9.9a	5.6a	0.5a
10^{-6}	0	97.2a	29.4a	10.2a	3.7b	0.3ab
10^{-5}	0	97.7a	12.8c	5.9b	3.4b	0.2ab
10^{-7}	10^{-7}	95.9ab	29.6a	10.3a	4.2ab	0.3ab
10^{-6}	10^{-6}	93.4ab	24.9b	7.7a	3.9ab	0.1ab
10^{-5}	10^{-5}	93.8ab	13.7c	2.4c	2.9b	0.04b

[a] DM = differentiation medium.
[b] RM = regeneration medium.
[c] Means in columns with the same letters are not significantly different at $p = 0.05$.

tration, because fewer calluses regenerated a high number of green shoots. These results show that in *P. pratensis* no benefit of ABA on plant regeneration was found. This is in contrast to the literature in which stimulation of ABA on plant regeneration was found for potato (Shepard 1980), wheat (Qureshi et al. 1989) and maize (Close & Ludeman 1987).

As compared to the control more calluses formed roots when 10^{-6} and 10^{-5} M ABA was added to the differentiation medium only. When ABA was also added to the regeneration medium, the percentage of calluses forming roots was intermediate, but the number of roots per callus was strongly reduced.

In an earlier paper on *P. pratensis* tissue culture differences in regeneration ability of calluses induced on immature inflorescences and mature seeds were described (Van der Valk et al. 1989). Inflorescence-derived calluses showed a much higher regeneration ability than the seed-derived calluses but regeneration was found to occur mainly via organogenesis. Somatic embryos were abundantly formed in these cultures and although many had a normal morphology, they failed to germinate and develop further into shoots. The present report shows that the addition of ABA did increase the percentage of calluses forming somatic embryos, but germination of the somatic embryos was unaffected by ABA.

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