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Brief Note

Plant regeneration after long term callus culture in clones of *Asparagus officinalis* L.*

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ABSTRACT: Callus growth and plant regeneration from long-term callus cultures were studied in two elite clones of *Asparagus officinalis* cv. Argenteuil, to establish a suitable protocol for a prospective *in vitro* selection program. Callus initiation and growth was evaluated on MS medium with 3% sucrose, 0.9% agar, 1 mg.l^{-1} kinetin, and three levels of 2,4-D. The highest callus relative growth was obtained on medium with 1.5 mg.l^{-1} 2,4-D and 1 mg.l^{-1} kinetin. Shoot primordia (SP) induction from >18-months-old calluses was evaluated on several media; the highest percentage of SP induction (89%) and average number of SP per callus (8.6) were obtained with clone '265' on MS medium with 5 mg.l^{-1} 2iP, 1 mg.l^{-1} IAA, 3% sucrose and 0.9% agar. The highest percentage of root induction (100%) was achieved with clone '265' on MS medium with 0.1 mg.l^{-1} kinetin, 0.1 mg.l^{-1} NAA, 1.32 mg.l^{-1} ancymidol, 7% glucose and 0.8% agar. Important medium x genotype interactions were detected, pointing to the need of adjusting this and other *in vitro* protocols for specific asparagus genotypes.

Abbreviations: 2,4-D: 2,4-dichlorophenoxyacetic acid; 2iP: 2-isopentenyladenine; BA: 6-benzylaminopurine; FFW: final fresh weight; IAA: indoleacetic acid; IFW: initial fresh weight; MS: Murashige and Skoog (1962); NAA: 1-naphtaleneacetic acid; PGRs: plant growth regulators; PVP: polyvinylpyrrolidone; SP: shoot primordia.

Introduction

'Crown and root rot', a disease caused by a fungal complex of *Fusarium* species, is the main cause of asparagus decline in the world (Dan and Stephens, 1995). Conventional breeding of resistant cultivars is a slow process that could be accelerated if more efficient selection methods were available. In other pathosystems, genetic materials with increased disease resistance were generated through *in vitro* selection in presence of pathogen toxins (in Jin *et al.*, 1996). In asparagus, the feasibility of *in vitro* selection for resistance to *Fusarium* toxins is scarcely documented. Selection methods exploit the occurrence of somaclonal variation induced during long-term callus cultures. However, a suitable protocol for regenerating whole plants from surviving long-term calluses must be available (Duncan, 1997). In this regard, organogenesis has been suggested as the most adequate pathway for preserving somaclonal variation (Duncan, 1997).

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There is a wide variety of published protocols for asparagus callus culture and organogenesis from callus (Harada, 1989; Reuther, 1990, Dan and Stephens, 1995; among others). However, they were developed for particular genetic materials, and are not broadly applicable, as has been our experience with selected genotypes of cv. Argenteuil (unpublished data). Thus, the objective of this work was to develop an efficient protocol for (1) long-term callus maintenance and growth and (2) plant regeneration via organogenesis from callus, that could be utilized in future *in vitro* selection in our breeding program.

Materials and Methods

Two staminate elite clones (genotypes) of cv. Argenteuil, '265' and '357', were used for all experiments. In all of them, the experimental unit was one test tube, and a completely randomized design was used. Callus induction was evaluated in three separate experiments with ten replications each. Internodal spear sections (2 mm long), excised from micropropagated plants, were cultivated in the dark at 26ºC for 60 days in 110 x 15 mm test tubes with Murashige and Skoog's (1962) basal medium (MS) plus 3% sucrose, 0.9% agar (Sigma), and combinations of plant growth regulators (PGRs; not shown). Using similar culture conditions, the best three of these combinations, 1 $mg.l^{-1}$ kinetin + 1.5, 5 or 10 $mg.l^{-1}$ 2,4-D, were tested in a callus growth assay with 23 replications. The initial (IFW) and final (FFW) callus fresh weights were recorded for each of two subcultures. Callus relative growth was calculated for each subculture as [(FFW-IFW)/IFW] and analyzed through standard ANOVA and Tukey test for mean comparison.

Five consecutive experiments were performed for designing a suitable protocol for shoot primordia (SP) induction from callus. The first was carried out with 18-months-old calluses, and each subsequent experiment was performed with calluses 2 1/2 months older than the used previously. The experiments consisted in the culture of callus pieces in test tubes (experimental unit=one test tube) with different SP induction media (Table 1) in the dark at 26ºC. Sixty days after culture initiation, the percentage of calluses with SP was determined on 20-25 replications per treatment. In the second experiment, the number of SP per callus was recorded as well. Log-linear models and standard ANOVA were used for analyzing the percentage of calluses with SP and the number of SP per callus, respectively.

TABLE 1.

Clones and media components evaluated in five consecutive experiments for adjusting shoot primordia induction from callus.

		Media components*								
					Auxin		Cytokinin			
Experiment	Clones	MS salts	Sucrose (%)	Agar (%)	Concentrations ($mg.l^{-1}$)	Types ($mg.l^{-1}$)	Concentrations	Types	Antioxidants treatments*	Number of
1	265 and 357	full strength	3	0.9	0 and 1	IAA	1, 5 and 10	BA, kinetin and 2iP	-	36
2	265 and 357	50% and full strength	2 and 3	0.9	0 and 1+	IAA	1 and 5	2iP	-	24
3	357x	Full strength	3	0.5, 0.7 and 0.9	1	IAA and NAA	5	2iP	-	6
4	357x	Full strength	3	0.5	0 and 1	IAA and NAA	5	2iP	None, 2 $mg.l^{-1}$ $AgNO_3$ and 0.1 $g.l^{-1}$ citric acid + 0.15 $g.l^{-1}$ ascorbic acid	9
5	357x	Full strength	3	0.5	0, 0.5 and 1	IAA and NAA	5	2iP and kinetin	None, 1 $g.l^{-1}$ PVP and 0.1 $g.l^{-1}$ activated charcoal	30

* Treatments were constituted by combining concentrations and types of media components in a factorial arrangement. Only one type of auxin, cytokinin or antioxidant was used in a given treatment.

+ 1 $mg.l^{-1}$ IAA was combined only with 5 $mg.l^{-1}$ 2iP (and not with 1$mg.l^{-1}$ 2iP).

x Twenty five calluses of clone 265, cultivated in MS salts with 3% sucrose, 0.5% agar, 1 $mg.l^{-1}$ IAA and 5 $mg.l^{-1}$ 2iP, were included as controls.

All calluses in which SP were induced in the first experiment were cultivated for 60 days on '85' medium (Daorden, pers. comm), to promote shoot and minicrown growth. This medium was composed of MS basal salts, '85' vitamins (1 mg.l^{-1} thyamine, 1 mg.l^{-1} nicotinic acid, 1 mg.l^{-1} pyridoxin, 1 mg.l^{-1} calcium panthotenate, 1 mg.l^{-1} glycine, 100 mg.l^{-1} myoinositol and 0.01 mg.l^{-1} biotin), 0.2 mg.l^{-1} kinetin, 0.2 mg.l^{-1} NAA, 0.1 mg.l^{-1} BA, 0.1 mg.l^{-1} ancymidol, 3% sucrose and 0.7% agar. The derived plantlets were randomly assigned to eight rooting media, constituted by combining two carbon sources -7% sucrose or glucose-, with two gelling agents -0.8% agar or gellan gum-, and two basal rooting media, (1) '102' (Daorden, pers. comm.), composed of MS salts, '85' vitamins, 0.1 mg.l^{-1} kinetin, 0.5 mg.l^{-1} ancymidol and 0.2 mg.l^{-1} NAA, and (2) 'DES' (Desjardins et al., 1987, with modifications), composed of MS salts, 0.1 mg.l^{-1} kinetin, 1.32 mg.l^{-1} ancymidol and 0.1 mg.l^{-1} NAA. After 45 days, the percentage of plantlets with healthy roots was determined on 20 replications; this variable was statistically analyzed using log-linear models.

Results

Statistical analysis of callus relative growth detected significant contributions of clones and 2,4-D levels. Calluses of '357' showed, on average, a higher relative growth than calluses of '265' in both subcultures ($p<0.05$). Callus relative growth diminished ($p<0.05$) with increased levels of 2,4-D, mainly in subculture 1. In both clones, a high proportion of calluses was of desirable appearance at all three 2,4-D levels, except for calluses of '357' on medium with 10 mg.l^{-1} of this auxin, which were compact and exhibited browning. Based on these results, the medium supplemented with 1.5 mg.l^{-1} 2,4-D and 1 mg.l^{-1} kinetin was selected for further inducing callus initiation and growth.

For adjusting SP induction from callus, five consecutive experiments were performed because poor results had been previously obtained after following several published protocols (Harada, 1989; Reuther and Becker, 1987 in Reuther, 1990; Reuther, 1990; Dan and Stephens, 1995). In experiment 1, SP induction from calluses varied between 0 and 62.5% in '265', depending on the culture medium; in contrast, no SP induction was observed in calluses of '357', that exhibited a compact structure and browning. Statistical analyses, performed only for data of '265', detected a significant contribution of the second order interaction; thus, the effects of each factor were evaluated for each of the levels of the remaining. The highest percentages of SP induction were found, in general, in media with 2iP; no clear trends were observed in relation to cytokinin concentration (Fig. 1). The effect of the addition of IAA depended on the type and concentration of cytokinin. When 1 mg.l^{-1} IAA was used in combination with any of the three levels of 2iP or with 5 mg.l^{-1} kinetin, SP induction was increased ($p<0.05$) in comparison with the observed in the same media without IAA, while the opposite was found when IAA was used in combination with 1 or 10 mg.l^{-1} kinetin or any of the three levels of BA. The best three out of the 18 PGR combinations evaluated in experiment 1 were selected for performing experiment 2.

Shoot primordia induction from callus was observed in almost all treatments of experiment 2. However, there were important differences between genotypes: depending on the culture medium, the percentage of SP induction varied between 39 and 89% in '265' and between 0 and 43.8% in '357', and the average num-

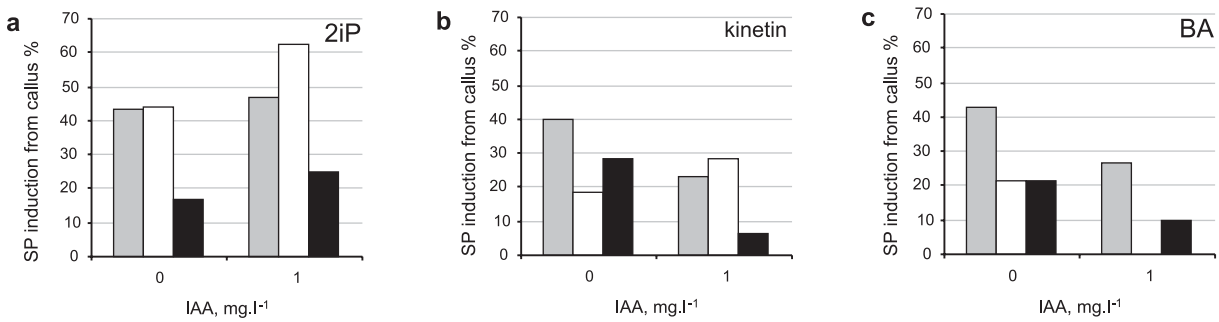

FIGURE 1. Average shoot primordia induction from callus of '265' in experiment 1. Cytokinins: a) 2iP; b) kinetin; c) BA. Cytokinin concentrations: 1 mg.l^{-1} (grey); 5 mg.l^{-1} (white); 10 mg.l^{-1} (black).

ber of SP per callus ranged between 2.4 and 8.6 in '265' and between 1 and 6.5 in '357'.

Significant contributions ($p<0.05$) to the statistical model of the percentage of SP induction were given only by main effects. On average, '265' showed a higher percentage of SP induction than '357'; furthermore, calluses of '357' exhibited the same appearance as in the previous experiment. The reduction in the concentrations of MS salts from 100 to 50%, and of sucrose from 3 to 2%, negatively affected SP induction. Conversely, the increase in both the concentration of 2iP from 1 to 5 $mg.l^{-1}$ and of IAA from 0 to 1 $mg.l^{-1}$ enhanced SP induction. The only significant contribution ($p<0.05$) to the statistical model of the number of SP per callus was given by the MS salts concentration; the highest value was observed when the full strength (100%) was used.

The highest percentages of SP induction from calluses of both '265' (62.5% in experiment 1 and 89% in experiment 2) and '357' (43.8% in experiment 2) were achieved with full strength MS basal salts supplemented with 5 $mg.l^{-1}$ 2iP, 1 $mg.l^{-1}$ IAA, 3% sucrose and 0.9% agar. Thus, this culture medium was utilized as a control in further experiments, carried out with '357'.

In experiment 3, the percentage of SP induction from callus varied between 0 and 31.25% in '357', depending on the culture medium, and was 52% in '265' (control). Only agar concentrations significantly contributed to the statistical model; the lowest concentration, 0.5%, enhanced SP induction in comparison with the remaining. However, generalized callus browning was observed in '357', as described earlier. In experiments 4 and 5, neither was SP induction from calluses of '357' attained, nor callus browning avoided, even with the use of antioxidants. On the other hand, SP induction from calluses of '265' was 41% in experiment 4 and 30% in experiment 5.

The percentage of root induction in '265' ranged between 0 and 100%, depending on the culture medium, but was not evaluated in '357' because few plantlets were available. The utilization of gellan gum resulted in a significantly lower percentage of root induction than the achieved with agar ($p<0.05$). The remaining main effects were analyzed jointly because a significant interaction between them was detected. When '102' was the basal rooting medium, the percentage of root induction was higher with sucrose than with glucose: the opposite response was obtained in 'DES' basal rooting medium. On the other hand, the average percentage of root induction was higher in 'DES' (67.25%) than in '102' (59.25%). The best percentage of root induction (100%) was obtained with 'DES' basal rooting medium, 7% glucose and 0.8% agar. This medium was preliminarly selected and tested in >100 plantlets of each of the two clones; on average, 45% and 65% root induction were obtained with '357' and '265' respectively.

Discussion

The aim of this study was to establish and characterize an *in vitro* culture system in asparagus for using in prospective long-term *in vitro* selection programs for resistance to *Fusarium* toxins. The medium selected for callus induction and growth is now routinely used for maintaining fast growing, healthy calluses of these and other clones of cv. Argenteuil in our laboratory.

Important interactions between IAA and the type and level of cytokinin occurred in SP induction from calluses of '265' (see experiment 1). The subsequent experiments showed conspicuous differences between clones concerning percentage of SP induction, number of SP per callus and callus appearance. Additionally, a loss of organogenic capacity was observed in both clones after the second SP induction experiments. Considering SP induction from calluses in the control medium, the only difference between experiments was callus age: as previously stated, experiment 1 was carried out with 18-months-old calluses, and each subsequent experiment was performed with calluses 2 1/2 months older than the used previously. The adjustment of SP induction accompanied the development of a long-term *in vitro* selection program for callus resistance to *Fusarium* toxins in our laboratory (unpublished data), and the utilization of calluses of similar age to the ones under selection was deemed convenient. According to our results, plant regeneration should be attempted in calluses approximately 20 months old.

In conclusion, an *in vitro* culture system for plant regeneration after long term callus culture is now available for two elite clones of *Asparagus officinalis* cv. Argenteuil. Although the medium selected for callus induction and growth is of broad application, the conditions for plant regeneration would have to be tested and, likely, adjusted for other genetic materials due to the presence of genotype x medium interactions.

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References

Dan Y, Stephens CT (1995). The development of asparagus somaclones with high levels of resistance to *Fusarium oxysporum* f. sp. *asparagi* and *Fusarium proliferatum*. Plant Dis 79: 923-927.

Desjardins Y, Tiessen H, Harney PM (1987). The effect of sucrose and ancymidol on the *in vitro* rooting of nodal sections of asparagus. HortScience 22: 131-133.

Duncan RR (1997). Tissue culture: induced variation and crop improvement. Adv Agron 58: 201-240.

Harada T (1989). Studies on organogenesis of asparagus (*Asparagus officinalis* L.) tissues and its utilization. Memoirs of the Faculty of Agriculture, Hokkaido University 16: 301-346.

Jin H, Hartman GL, Huang YH, Nickell CD, Widholm JM (1996). Regeneration of soybean plants from embryogenic suspension cultures treated with toxic culture filtrate of *Fusarium solani* and screening of regenerants for resistance. Phytopathology 86: 714-718.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissues cultures. Physiol Plant 15: 473-497.

Pontaroli AC, Camadro EL (2001). Selección de callos de genotipos selectos de *Asparagus officinalis* cv. Argenteuil por comportamiento frente al filtrado tóxico de *Fusarium* (Abst). J Basic Appl Genet 14: 112.

Reuther G (1990). Histological and cytological selection of somaclonal and induced variants of asparagus derived from callus cultures. Acta Hort 280: 385-393.