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Callus formation and plant regeneration through direct culture of isolated pollen of *Hordeum vulgare* cv. 'Sabarlis'

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Summary. Pollen calli and plantlets of *Hordeum vulgare* cv. 'Sabarlis' were obtained through direct pollen culture without pretreatment of spikes or preculture of anthers. Isolated immature pollen grains were cultured first in a 0.3 M mannitol solution or a C1 basal medium (Chen et al. 1979) supplemented with 0.3 M mannitol but without sucrose for 5–7 days, then transferred into a C1 medium containing 6% sucrose, 3 mM glutamine and 5 mM m-inositol. After a 3 week culture period small pollen calli derived from the pollen grains were transferred into a growth medium comprising C1 basal medium supplemented with 250 mg/l lactalbumin hydrolysate and 0.5 mg/l kinetin. For shoot regeneration, vigorously growing calli were transferred onto agar-solidified MS medium (Murashige and Skoog 1962) containing 3% sucrose, 2 mg/l benzyladenine and 0.5 mg/l indole-3-acetic acid. The ratio of green plants to albino was approximately 1:2.2.

Key words: *Hordeum vulgare* – Direct pollen culture – Callus formation – Plant regeneration

Introduction

In vitro production of haploid plants from pollen (mechanically isolated from anthers or shed from anthers floated on liquid medium) has been attempted in various cereals: rye (Wenzel et al. 1975), barley (Xu and Sunderland 1981, 1982), rice (Chen et al. 1980) and wheat (Wei 1982). In these cases, however, calli were obtained from the isolated pollen by pretreating the spikes or preculturing the anthers. The induction of callus or plant regeneration through direct pollen culture in cereals has not yet been reported. Recently, successful plant regeneration through direct pollen culture was reported in a *Nicotiana* species (Ghandimathi 1982; Imamura et al. 1982; Kyo and Harada 1985). According to these reports, freshly isolated pollen was cultured first in a sucrose-free medium for several dyas, then transferred into a suitable liquid medium containing sucrose. We have applied this method to pollen culture of 'Sabarlis' barley and obtained some successful results.

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Materials and methods

Hordeum vulgare cv. 'Sabarlis' plants were grown outdoors in pots during the normal growing season. Spikes, of which the length of apical awns were about 2–5 cm, were collected. Most of immature pollen grains in these spikes were at an early to middle bicellular stage of the development.

Initial culture

The spikes were surface sterilized with 70% ethanol for about 30 s, then the anthers were excised aseptically from the spikelets at the middle area of the spikes. The spikelets located in the upper and lower areas were discarded so as not to increase the heterogeneity of the pollen population. Immature pollen grains were isolated by gently pressing the anthers, now placed in 0.3 M mannitol solution, with an internal pestle or injector and filtering through a nylon sieve (pore size 100 μm). The pollen grains were rinsed twice in 0.3 M mannitol by centrifugation at 150 xg for 1 min, then suspended in 0.3 M mannitol or C1 medium supplemented with 0.3 M mannitol without sucrose. The density of the pollen suspension was about $0.5–1 \times 10^5$ grains/ml. Four ml aliquots of the suspension were placed in Petri dishes (6 cm in diameter). The dishes were sealed with parafilm and cultured at 25 °C in the dark.

Re-culture

After an initial culture period which varied in duration, for the pollen grains were rinsed by centrifugation and resuspended in

Table 1. Effect of different media on the initial culture of pollen callus formation in *Hordeum vulgare* cv. 'Sabarlis'

Media	I	II	III	IV	V
	Distilled water	C1[a] with 9% sucrose	0.3 M mannitol	0.3 M mannitol with C1	0.3 M mannitol with C1 and 9% sucrose
No. of pollen calli per petri dish[a]	0	0	57	86	0

[a] C1 basal medium without sucrose
[b] Three weeks after the beginning of the re-culture, pollen calli larger than 1 mm were counted

Table 2. Effect of the initial culture period on pollen callus formation in *Hordeum vulgare* cv. 'Sabarlis'

Days of the initial culture	0	1	2	3	5	7	9
No. of pollen callus per petri dish							
Exp. I	0	0	0	7	54	98	41
Exp. II	0	0	0	9	44	59	–

[a] Three weeks after the beginning of the re-culture, pollen calli larger than 1 mm were counted

Table 3. Effect of sucrose in the re-culture medium on pollen callus formation in *Hordeum vulgare* cv. 'Sabarlis'

Concentration of sucrose (%)	0	1	2	3	6	9	12
No. of pollen calli per petri dish	0	8	14	46	74	52	7

[a] Three weeks after the beginning of the re-culture, pollen calluses larger than 1 mm were counted

C1 liquid medium containing 3 mM glutamine and 5 mM m-inositol (Xu and Sunderland 1981). Hereafter this culture is referred to as the re-culture. The pollen density and culture conditions were same as those described above.

Culture of pollen calli

Pollen calli stopped growing and turned brown if left for more than 4 weeks in the re-culture medium, thus losing the capacity of regeneration. Therefore, after about 3 weeks of re-culture, pollen calli larger than about 1 mm in diameter were transferred by pipette to a medium consisting of C1 basal medium supplemented with 250 mg/l lactalbumin hydrolysate and 0.5 mg/l kinetin (Wei 1982). Hereafter we designate this culture medium as the growth medium. The cultures were placed under dim light conditions. Other conditions were same as those described above.

Regeneration of plants

About 2 weeks after transfer to the growth medium, vigorously growing calli larger than about 3 mm in diameter were again transferred to a solid medium comprised of the basal constituents of MS medium (Murashige and Skoog 1962), containing 0.5 mg/l indole-3-acetic acid (IAA), 2 mg/l benzyladenine (BA), and 3% agar. Hereafter we designate this medium the regeneration medium. Shoots which developed on this medium were subsequently transferred to the rooting medium which consisted of half strength MS constituents, with 2% sucrose, 0.5 mg/l IAA, 0.1 mg/l Kinetin and 1% agar. Culture conditions for regeneration were same as those described above. All culture media were adjusted to pH 5.8 by 1N NaOH. The viability of pollen was examined by fluorescein diacetate staining.

Results

Callus formation

Immature pollen grains isolated directly from anthers without any pretreatment or preculture were cultured in different media. The effects of different media on the initial culture of pollen callus formation is shown in Table 1. Neither multicellular pollen nor callus was observed when immature pollen grains were cultured first in plain water or media with 9% sucrose. However, multicellular pollen and calli were formed during re-culture when 0.3 M mannitol with or without C1 basal medium components except for sucrose were used as the initial culture medium.

The effects of initial culture period on callus formation are shown in Table 2. Neither multicellular pollen nor callus was formed when the period of the initial culture was shorter than 3 days. Multicellular pollen and calli were observed when the initial culture lasted longer than 3 days.

After an initial culture of 7 days, the pollen was transferred into the re-culture medium containing sucrose at various concentrations and the effect of sucrose concentration on callus formation was examined (Table 3). Pollen calli were formed in the media containing sucrose, and its optimum concentration was 6%. When sucrose was omitted from the medium, callus formation was not observed but a small number of multicellular pollen grains were sometimes found (data not shown).

The process of callus formation from pollen during the re-culture is shown in Fig. 1.

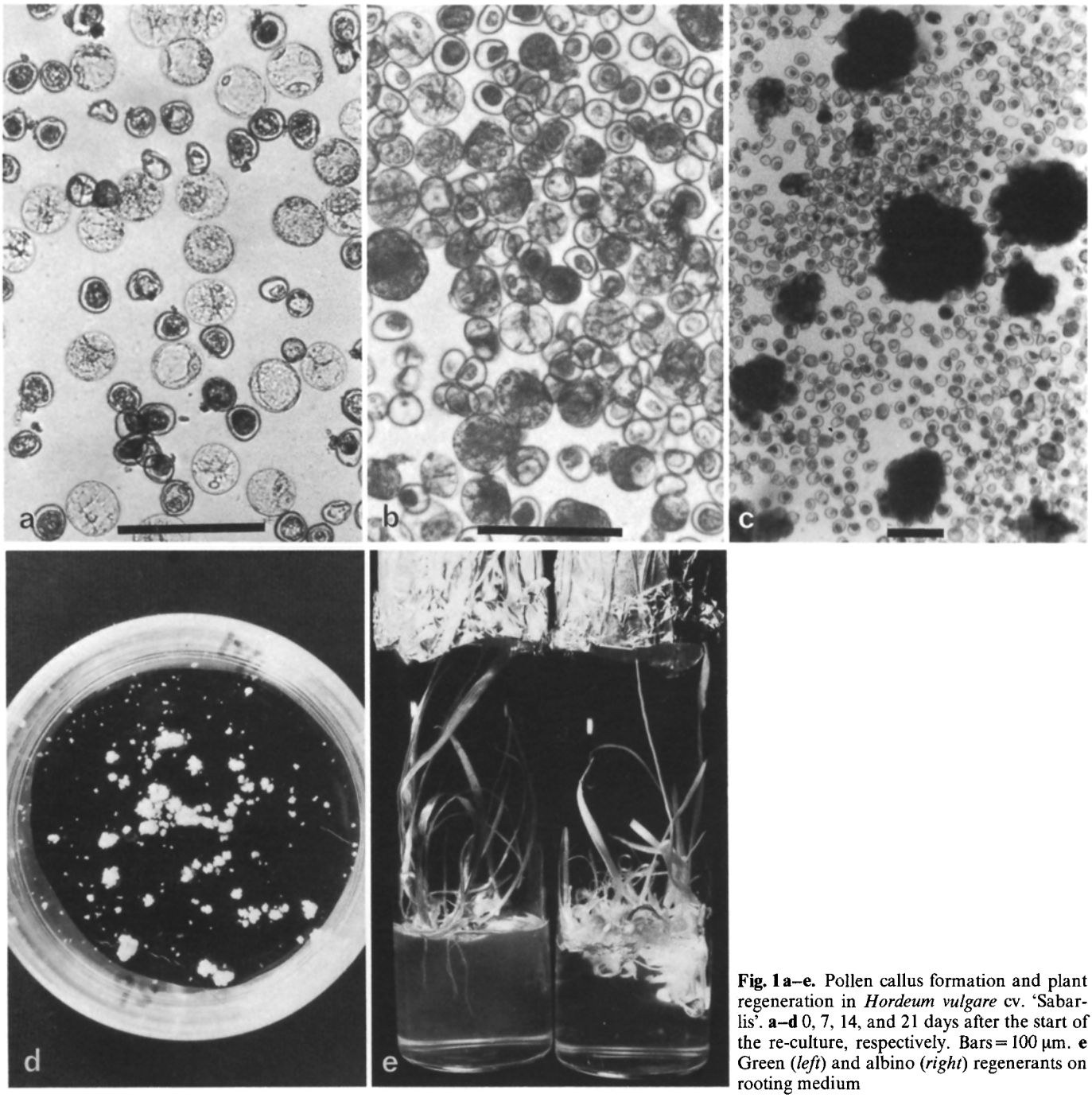

Fig. 1 a–e. Pollen callus formation and plant regeneration in *Hordeum vulgare* cv. 'Sabarlis'. **a–d** 0, 7, 14, and 21 days after the start of the re-culture, respectively. Bars = 100 µm. **e** Green (*left*) and albino (*right*) regenerants on rooting medium

Plant regeneration

After about 3 weeks of culture in the re-culture medium, the pollen calli were transferred into the growth medium or fresh re-culture medium. After about 2 weeks, calli larger than 3 mm in diameter were transferred onto the regeneration medium, and the frequency of green shoot regeneration was examined (Table 4). The frequency of shoot regeneration from pollen calli which were formed in the growth medium was roughly 60%, and the ratio of green plants to albino was approximately 1:2.2. These results are better than that those obtained with pollen calli which developed in the re-culture medium (Table 4). Eighty-five percent of the green shoots developed roots with the rooting medium (Fig. 1 e).

Table 4. Shoot regeneration frequency in pollen calli of *Hordeum vulgare* cv. 'Sabarlis' cultured in the re-culture medium or the growth medium

Medium for callus development	No.		
	Inoculated callus	Green shoot	Albino shoot
Re-culture medium	67	4 (5.9)[a]	23 (34.4)
Growth medium	74	14 (18.1)	31 (41.9)

[a] Figures in parentheses are the percentages based on the number of inoculated calli

Discussion

The results presented above indicate that the direct pollen culture method which was developed initially for *Nicotiana* (Gahandimathi 1982; Imamura 1982; Kyo and Harada 1985) are also applicable to 'Sabarlis' barley.

The appropriate pollen stage for obtaining plant regeneration was not entirely confirmed although most of cultured pollen grains were at an early to middle bicellular stage, which were not adequate stages for either anther culture or previously reported pollen culture with pretreatment. The pollen at the unicellular to mitotic stage which were adequate for anther culture could not be used because the viability was completely lost during the isolation process from the anthers. The late bicellular to mature pollen grains completely lost their viability during the initial culture with all media used in this study. For the pollen at the suitable stage, namely, the early to middle bicellular pollen, the viability at the beginning and the end of the initial culture was 10% and 8%, respectively, in the best case.

In the media which were unsuitable for the initial culture, pollen viability was completely lost during the culture period. The differences between media I and III, and media IV and V (Table 1) indicate that a suitable osmotic pressure and the absence of sucrose (9%) allow the cultured pollen to remain alive.

As shown in Table 4, the difference in the components between two media, namely, the re-culture medium (C1 medium containing 3 mM glutamine and 5 mM m-inositol) and the growth medium (C1 basal medium supplemented with 250 mg/l lactalbumin hydrolysate and 0.5 mg/l kinetin) resulted in the differences in regeneration capacity.

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References

Chen Y, Wang R, Tian W, Zuo Q, Zeng S, Lu D, Zhang G (1980) Studies on pollen culture in vitro and induction of plantlets. *Oryza sativa* Subsp. Keng. Acta Genet Sin 7:45–54

Ghandimathi H (1982) Direct pollen culture of *Nicotiana tabacum.* In: Fujiwara A (ed) Plant tissue culture 1982. Maruzen, Tokyo, pp 527–528

Imamura J, Okabe E, Kyo M, Harada H (1982) Embryogenesis and plantlet formation through direct culture of isolated pollen of *Nicotiana tabacum* cv. 'Samsun' and *Nicotiana rustica* cv. 'Rustica'. Plant Cell Physiol 23:713–716

Kyo M, Harada H (1985) Studies on conditions for cell division and embryogenesis in isolated pollen culture of *Nicotiana rustica.* Plant Physiol 79:90–94

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassey with tobacco tissue culture. Physiol Plant 15:473–479

Sunderland N, Xu ZH (1982) Shed pollen culture in *Hordeum vulgare.* J Exp Bot 33:1086–1095

Wei ZM (1982) Pollen callus culture in *Triticum aestivum.* Theor Appl Genet 63:71–73

Wenzel G, Hoffmann F, Potrykus I, Thomas E (1975) The separation of viable rye microspore from mix populations and their development in culture. Mol Gen Genet 138:293–297

Xu ZH, Sunderland N (1981) Glutamine, inositol and conditioning factor in the production of barley pollen callus in vitro. Plant Sci Lett 23:161–168