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Plant regeneration by pollen embryogenesis from cultured whole spikes of barley (*Hordeum vulgare*)

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Summary. Pollen embryogenesis and subsequent plant regeneration have been established from cultured whole barley spikes in agitated N6 liquid medium (Chu 1978) containing high levels of 2,4-D, Ficoll and potato extract. Microspore division within the anthers and subsequent embryogenic development were obtained in medium containing high amounts of reduced nitrogen with Zeatin, NAA and BAP (all at 0.5 mg/l levels, pH 6.2). Once embryoids were formed in the liquid medium, they produced secondary embryoids from the scutellum and subsequently plants on MS (Murashige and Skoog 1962) agar medium containing BAP and NAA. The ratio of green plants to albino was 1 : 8.7.

Key words: *Hordeum vulgare* – Pollen embryogenesis – Regeneration – Whole spike – Haploidy

Introduction

Plant regeneration from in vitro cultures originating from multicellular explants has been achieved in all major cereal crops (Vasil 1983; Han 1985; Heberle-Bors 1985; Lörz et al. 1986). Innovations in in vitro culture technology, such as anther (microspore) culture, can be incorporated into plant breeding programs. This technique, combined with protoplast fusion, plant regeneration from single cells, provides the necessary framework for profound work on cell genetics and genetic modifications in higher plants (Potrykus et al. 1979; Han 1985; Miah et al. 1985). In barley, successful regeneration has been obtained from a number of explant sources including apical meristem derived callus (Cheng and Smith 1975; Weigel and Hughes 1985) and cultured barley anthers (Kasha and Kao 1970; Clapham 1973; Foroughi-Wehr et al. 1976; Sunderland and Huang 1985); from immature embryos (Dale and Deambrogio 1979; Hanzel et al. 1985; Wenzel et al. 1985; Goldstein and Konstad 1986; Lörz et al. 1986) and from microspore culture (Wei et al. 1986). The only report of barley spike culture (Wilson 1977) had limited success with plant regeneration. Recent success in isolated microspore embryogenesis in wheat (Datta and Wenzel 1987) and barley (in preparation) has made it easier to elucidate a better regeneration system of pollen plants via embryogenesis from barley spike culture. This has been a long desired objective as it avoids the plastid mutation and chimeric development of plants.

Materials and methods

Plant material

Experiments were carried out with one winter barley ('Igri') and one F_1 hybrid winter barley of a breeders line ('Franka' × 'Igri'). All plants were grown in the greenhouse. Tillers containing microspores at early uninucleate stage were clipped off at ground level and kept at 5 °C for 12 days following the principle of cold treatment (Huang and Sunderland 1982). Sterilization of the spike was done with 1.8% sodium hypochlorite for 7 min with constant stirring followed by washing three times with deionized water. Each spike was placed in 15 ml liquid medium in 100 ml Erlenmeyer flasks and the cultures were shaken orbitally (110 rpm) at 24 °C in the dark.

Media and culture condition

The media composition used in this study with the optimum concentrations of growth substances including phytohormones are given in Table 1, based on N6 (Chu 1978) and MS (Murashige and Skoog 1962) media. The initial medium used for the induction of microspore divisions (started after 6 days) within the anthers is designated as BM1. The replenishment of the cultures containing dividing microspores were done after two weeks of culture with another liquid medium designated as BM2. Embryogenic structures formed on BM2 medium were transferred to regeneration agar medium after 6–8 weeks of culture (RM). Green plants raised on RM medium were transferred to the field after 3 months of culture.

Results and discussion

The data scored here and shown in the results (Table 2) were obtained on the two genotypes. The results showed that spike cultures of both genotypes could produce embryogenic calli in the Ficoll medium. In both genotypes the combined use of Ficoll and 2,4-D within their optimal range resulted in embryogenic calli. Kao (1981) introduced Ficoll in the medium; Huang and Sunderland (1982) showed successful use of cold stress for barley anther culture; Xu et al. (1981) showed the utility of conditioning medium for pollen callus formation; Köhler and Wenzel (1985) have tried to find out the factor responsible for conditioning the medium. A modified method of conditioning the medium with the ovary as co-culture and Ficoll incorporated medium with cold stressed anthers were used for the microspore embryogenic plant formation in wheat (Datta and Wenzel 1987). A similar approach used in the cold treated barley spike showed a high frequency of embryogenic calli formation (Figs. 1–3).

The probability that these EC were of pollen origin is evident by the following observations – also in agreement with Wilson (1977): 1) unorganised growth from other tissues of the spike did not occur; 2) EC emerged directly from within the anther sacs; 3) callus production from the connective or anther walls was never detected in this or previous studies (Clapham 1973; Wilson 1977); 4) Individual responding anthers showed a high number of numerous microspore divisions and subsequent EC development (Figs. 3–4).

The initial culture medium (BM1) containing potato extract, Ficoll and 2,4-D and other growth substances shown in the Table 1 is the most critical factor in determining the embryogenic pathway of microspore divisions which was further accelerated on BM2 medium with a lower level of auxin, 0.5 mg/l NAA and cytokinins (BAP, Zeatin both at 0.5 mg/l). As a carbohydrate source, 6% (w/v) sucrose with 0.2% (w/v) myo-inositol and 0.5% (w/v) glucose have been found beneficial in the initial culture followed by reduced carbohydrates in general (Table 1) for plant formation. This has also been shown in other cereal cultures (Dunwell 1985; Datta and Wenzel 1987; Wei et al. 1986).

However, once the embryogenic calli formed on the BM2 medium, secondary embryoids derived from scutellum of primary embryoids and, subsequently, plants, were produced when transferred to simple modified MS medium containing reduced carbohydrates, BAP and NAA (Figs. 5–6). Plants were also obtained directly through the germination of such pollen embryoids on the same medium. The cultures of EC obtained on BM2 medium could be maintained on MS agar medium with 0.5 mg/l Zeatin, 0.5 mg/l BAP and 0.5 mg/l NAA for at least 6 months with a high regeneration capacity. Of the 155 plants regenerated, 16 were green, 137 albino and 2 variegated.

Elimination of the callus phase and the formation of embryoids directly from haploid pollen would be advantageous. Such a system would shorten the culture procedure and reduce the danger of plastid mutation and chimeric development of regenerants as there is a strong selection in favour of normal cells during the

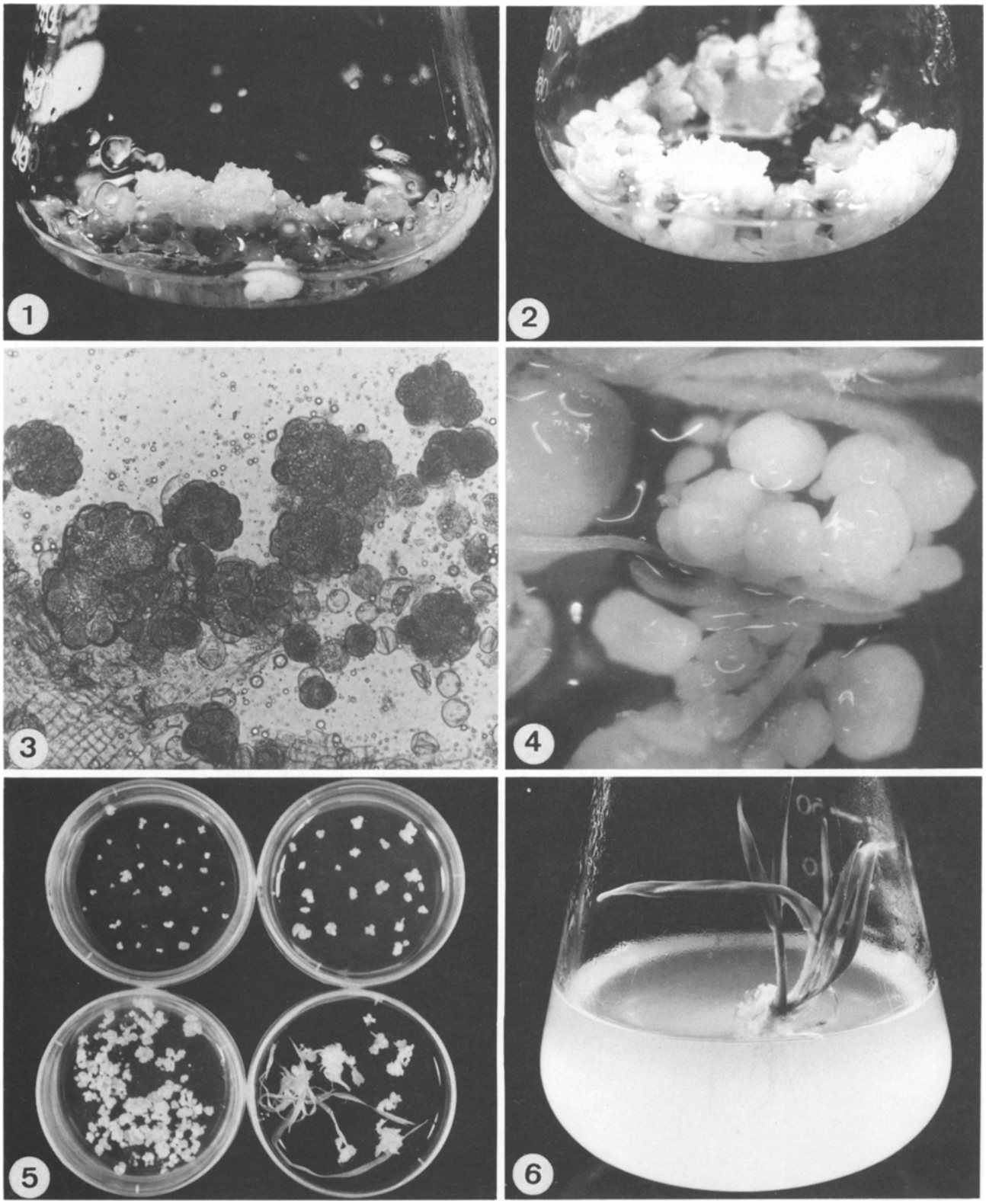
Table 1. Media composition for pollen plant formation from barley spike culture

Basal medium[a] +	Induction of microspore Dn. BM1 (mg/l)	Development of EC BM2 (mg/l)	Plant formation RM (mg/l)
Glutamine	160	160	–
Proline	50	50	–
2,4-D	5	1.0	–
NAA	–	0.5	1.0
BAP	–	0.5	1.0
Zeatin	–	0.5	–
Kinetin	0.5	–	–
Sucrose	60,000	60,000	30,000
Glucose	5,000	5,000	–
Myo-inositol	2,000	1,000	100
Casein-hydrol.	–	1,000	500
Lactalbumin hydr.			250
Ficoll	100,000	100,000	–
Potato extract	100,000	–	–
Agar	–	–	7,500
Activated charcoal	–	–	5,000
pH	5.8	6.2	5.8

[a] Basal media (BM1 and BM2) based on N6 and regeneration medium (RM) based on MS medium

Table 2. Plant regeneration via pollen embryogenesis from barley spike culture

Genotypes	No. of spike cultured (BM1)	% of response	No. of EC/Spike (BM2)	Plant formation	
				Albino (RM)	Green (RM)
'Igri'	110	50	82	69	9
F_1 ('Franka' × 'Igri')	80	60	67	70	7



Figs. 1–6. Barley spike culture and pollen plant formation via embryogenesis. **1** Spike cultured in liquid medium showing calli formation after 4–5 weeks of culture. **2** Same after 6 weeks of culture. **3** Embryogenic multicellular microcalli developed within anthers after 3 weeks of culture. **4** Further development leading to globular embryoids with distinct scutellum. **5** Growth and development of embryogenic calli leading to green and albino plant formation. **6** A green plant after 10 weeks of culture

process of embryogenesis (Day and Ellis 1985; Sunderland and Huang 1985; Datta and Wenzel 1987). The present study provides the possibility of high frequency pollen embryogenic plant formation by a simple method of whole barley spike culture. Being the simplest, the *hap* system deserves detailed investigation in the direction of maximizing the efficiency of haploid breeding in barley.

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