STUDIES ON PLANT CELL AND TISSUE CULTURE

III. *In vitro* Induction of Callus from Anther Culture of Forage Crops

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Introduction

Since Guha and Maheshwari (1964, 1966) succeeded in producing haploid plants by anther culture of *Datura innoxia in vitro*, a number of workers have attempted to apply anther culture to a wide species of plants. This is because haploid plants are generally recognized for their usefulness for various approaches in cytogenetics, genetics, and plant breeding works. Thus to the present, a considerable amount of success of producing haploid plants by anther culture have been reported for other species: e.g. *Nicotiana* species (BOURGIN and NITSCH, 1967; NAKATA and TANAKA, 1968; SUNDERLAND and WICKS, 1969 a, b), *Oryza sativa* (NIIZEKI and OONO, 1968; NISHI and MITSUOKA, 1969; HARN, 1969; GUHA *et al.*, 1970), *Brassica* (KAMEYA and HINATA, 1970), *Lolium multiflorum* × *Festuca arundinacea* (NITZSCHE, 1970), *Lolium multiflorum* and *Hordeum vulgare* (CLAPHAM, 1971) *Datura metel* (NARAYANASWAMY and CHANDY, 1971), *Atropa belladonna* (ZENKTELER, 1971), and *Aegilops* (KIMATA and SAKAMOTO, 1972).

The present paper gives the result of work aimed at inducing haploid plants of forage crops belonging to Gramineae and Leguminosae which is of considerable economic importance.

Materials and Methods

Nine species belonging to Gramineae were used; *Bromus inermis* (var. Elsberry), *Festuca arundinacea* (var. Grombalia), *Festuca pratensis* (var. Naïade), *Lolium hybridum* (Hybrid ryegrass I_o), *Lolium multiflorum* (var. Rina), *Lolium perenne* (var. Bocage), *Dactylis glomerata* (cv. Tsukisamu-Zairai), *Phleum pratense* (P. I. 58382 and var. Norin-Ichigo), and *Sorghum*

sudanense (var. California). Also, three species belonging to Leguminosae were used; *Lotus corniculatus* (cv. Empire), *Medicago sativa* (var. DuPuit), and *Trifolium pratense* (var. Alpilles and var. Triel).

The mineral salts and organic constituents of the media used in this study are the same as those described by MILLER (1963) or NITSCH and NITSCH (1969). Various concentrations of growth regulators such as 0.1–10.0 mg/l of IAA, 1.5–20.0 mg/l of 2, 4-D, 1.5–20.0 mg/l of NAA, and 0.1–10.0 mg/l of cytokinin (kinetin or 6-benzylaminopurine) were added to the basic medium singly or in various combinations. Each Medium was solidified with 10 g/l of Difco Bacto-agar, and the pH was adjusted to 6.0.

Florets or flower buds were dipped into 70% ethanol and washed several times with sterilized distilled water. They were then immersed in a 7% solution of sodium hypochlorite for about 3 minutes and thoroughly washed with sterilized distilled water. Anthers of each species were removed aseptically from the florets or the flower buds and they were then sown on each medium. The identification of the developmental stages of the anthers of each species was carried out by a microscopic examination of the meiocytes and anthers containing individualized uninucleate pollen grains were selectivly used in this study.

All cultures were incubated in the dark at 26±0.5°C.

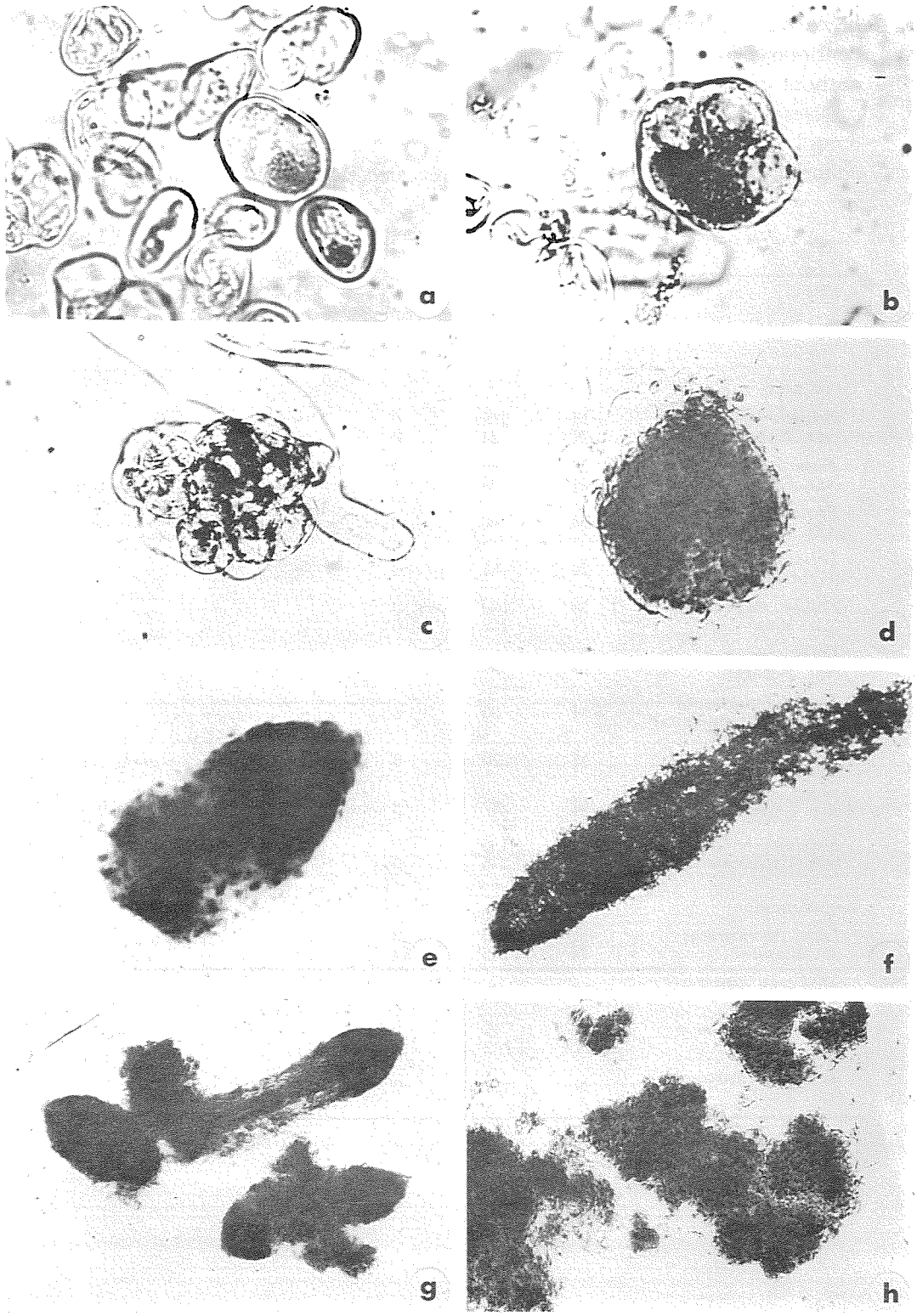
Results

Anther culture in Gramineae

Among nine species in Gramineae, two species, *Festuca arundinacea* and *Phleum pratense*, formed calluses on the cultured anthers after three to four weeks of incubation (Table 1). In *Festuca arundinacea* only 1 of 99 anthers formed a callus on the basic medium of NITSCH and NITSCH (1969) which contained a rather high concentration of growth regulators such as 10 mg/l of IAA and 10 mg/l of kinetin. The growth of the callus,

Fig. 1. Various stages in the formation of embryoids and calluses from pollen grains in cultured anthers of *Phleum pratense* (var. Norin-Ichigo).

a. Individualized uninucleate pollen grains. × ca. 650.
b. A spherical mass with several cells. × ca. 650.
c. A cluster with about 10 cells. × ca. 650.
d. Initiation of polarity. × ca. 400.
e. Establishment of polarity. × ca. 150.
f. Cotyledonary stage. × ca. 75.
g. Initiation of calluses from embryoids. × ca. 60.
h. Establishment of calluses from embryoids. × ca. 60.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however, was very slow and finaly appeared to cease after two months of continuous incubation. On the other hand, in *Phleum pratense* 5 of 270 anthers of var. Norin-Ichigo and 3 of 267 anthers of P. I. 58382 formed calluses vigorously on the basic medium of MILLER (1963) which contained 6 mg/l of NAA or 3 mg/l of 2, 4-D. Microscopic observations of the cultured anthers of these two species revealed various developmental stages

TABLE 1. Callus, shoot and root formation by anther culture of forage crops

Materials	Medium*	Number of anthers used	Number of anthers froming calluses	Shoot formation from callus**	Root formation from callus**	Origin of callus
Gramineae						
Bromus inermis (var. Elsberry)	M	135	0	−	−	
	N	24	0	−	−	
Festuca arundinacea (var. Grombalia)	M	150	0	−	−	
	N	99	1	−	−	germ cell
Festuca pratensis (var. Naïade)	M	90	0	−	−	
Lolium hybridum (Hybrid rygrass I_0)	N	81	0	−	−	
Lolium multiflorum (var. Rina)	M	180	0	−	−	
	N	108	0	−	−	
Lolium perenne (var. Bocage)	M	90	0	−	−	
Dactylis glomerata (cv. Tsukisamu-Zairai)	M	90	0	−	−	
Phleum pratense (var. Norin-Ichigo)	M	270	5	−	+	germ cell
Phleum pratense (P.I. 58382)	M	267	3	−	+	germ cell
Sorghum sudanense (var. California 23)	M	135	0	−	−	
	N	135	0	−	−	
Leguminosae						
Lotus corniculatus (cv. Empire)	M	75	29	+	+	somatic cell
	N	108	14	+	+	somatic cell
Medicago sativa (var. DuPuit)	M	90	14	−	+	somatic cell
Trifolium pratense (var. Alpilles)	M	45	1	−	−	somatic cell
	N	162	0	−	−	
Trifolium pratense (var. Triel)	N	27	0	−	−	

* M, basic medium of Miller; N, basic medium of Nitsch and Nitsch.

** −, no formation; +, formation.

from pollen grains to embryoids (Fig. 1 a, b, c, d, e, f). The pollen grains at the uninucleate stage firstly differentiated into globular masses of multicells and then developed to embryoids. Later, the embryoids developed distinct cotyledon and roots. However, complete plantlets were not developed from the embryoids and all of them differentiated into calluses (Fig. 1 g, h). Several root formations were observed on these calluses in continuous incubation.

Anther culture in Leguminosae

In two species, *Lotus corniculatus* and *Medicago sativa*, high frequencies of callus formations were generally observed on any media, while in *Trifolium pratense* the induction of the callus was quite rare on any media (Table 1). Microscopic observations of the cultured anthers of these three species indicated that some of the pollen grains swelled up enormously and occasionally hypertrophied examples were observed bursting from the cell walls. Also, pollen grains were frequently observed with numerous starch grains and thickened cell walls. However, there was no evidence that cell divisions or multicellular formations initiated from the pollen grains, and therefore, in the case of Leguminosae all of the calluses formed by the anther culture were considered to have originated from somatic tissues of the anthers such as connective tissues or filament residues rather than the germ cells.

Discussion

Two species belonging to the Gramineae, *Phleum pratense* and *Festuca arundinacea* formed a few calluses which were considered to have originated from the germ cells. On the other hand, *Lotus corniculatus* and *Medicago sativa* belonging to the Leguminosae formed calluses at high frequencies. However, it appeared that all calluses of the species in the Leguminosae probably originated from the somatic tissues rather than the germ cells. The causes of this difference of the callus formations between the Gramineae and the Leguminosae are still unclear. However, some possible answers may be given to explain the lack of the callus formation from the germ cells in the Leguminosae. In several studies, successful inductions of the calluses from pollen grain culture instead of anther culture have been reported for several species, namely *Ginkgo biloba* (TULECKE, 1953, 1957), *Torreya nucifera* (TULECKE and SEHGAL, 1963), *Ephedra foliata* (KONAR, 1963), *Brassica oleracea* (KAMEYA and HINATA, 1970), and *Petunia hybrida* (BINDING, 1972). In our study on pollen grain culture of *Lotus*

species, cell division was initiated and subsequently 2 to 3 celled pollen grains were formed, although no cell division was induced by anther culture (NIIZEKI and GRANT, 1971). These results seem to suggest that some of the factors involved in unsuccessful anther culture might be simply the suppression of some nutritional requirement used in the induction and the vigorous growth of the somatic callus, or the upsetting of the balance between endogenous and exogenous growth regulators, or the disappearance of some inhibitors which existed in the anther. However, it can not be concluded here as to which factor or factors are responsible for the cell division of the pollen grains.

NAKATA and TANAKA (1968), and NITSCH and NITSCH (1969) reported that the process of embryoid formation in the anther culture of *Nicotiana* species is similar to that in normal embryogenesis. Individualized pollen grains increase in size and form a round mass of cells. Then, the integuments of the pollen grains break up and liberate what may represent the globular stage of a normal embryo. Then, the "heart", "torpedo", and "cotyledonary" stages follow. Later, the embryoids germinate and give rise to complete plantlets. In contrast to *Nicotiana* species, in the present study on *Phleum pratense* and *Festuca arundinacea* the embryoids from the pollen grains differentiated into the calluses instead of germinating into complete plantlets. A study on *Nicotiana tabacum* indicated that the difference between the induction of plantlets or calluses from the pollen grains depends on the difference of growth regulators in medium (NIIZEKI 1972). On medium containing a low level of concentration of growth regulators, 0.1 mg/l of IAA and 0.1 mg/l of 6-benzylaminopurine, only haploid plantlets were induced directly from the pollen grains. On the other hand, both haploid plantlets and haploid calluses were induced on a medium containing a high level of concentration of growth regulators, namely 4.0 mg/l of IAA and 2.0 mg/l of 6-benzylaminopurine and only haploid calluses were induced on the medium containing 4.0 mg/l of NAA and 2.0 mg/l of 6-benzylaminopurine. No detailed observation in the present study was carried out on the mode of development of pollen grains affected by the type of media. Therefore, the similarities and the differences in the manner of differentiation arising from pollen grains between *Nicotiana* species and the species of Gramineae remain unclarified.

Summary

Six genera in Gramineae; *Bromus, Festuca, Lolium, Dactylis Phleum, Sorghum,* and three genera in Leguminosae; *Lotus, Medicago, Trifolium,*

were used in the present study on anther culture of forage crops.

In the Gramineae two species, *Festuca arundinacea* and *Phleum pratense*, formed several calluses from individualized uninucleate pollen grains in the cultured anthers. Microscopic observations indicated that the development of embryoids from the pollen grains occurred as in normal embryogenesis. However, all of the embryoids failed to germinate to complete plantlets but differentiated to the calluses.

Lotus corniculatus and *Medicago sativa* in the Leguminosae formed calluses on the cultured anthers at high frequencies with the exception of *Trifolium pratense*. Microscopic observations of the pollen grains in the cultured anthers indicated some morphological changes such as hypertrophy of the pollen grains and the pollen with numerous starch grains. However, there was no evidence that cell division or multicellular tissue had initiated from the germ cells. As a result, it was concluded that the calluses in the species of Leguminosae originated from somatic tissues of the anthers rather than from the germ cells.

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