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Somatic embryogenesis and plantlet regeneration in the genus *Secale*

1. Somatic embryogenesis and organogenesis from cultured immature embryos of five wild species of rye

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Summary. A tissue culture of five wild species of the *Secale* genus, i.e., *S. africanum* (Stapf.), *S. ancestrale* (Zhuk.), *S. kuprianovii* (Grossh), *S. segetale* (Rosher.), and *S. vavilovii* (Grossh), from immature embryos of sizes (stages) varying between 1.0 mm to 3.0 mm, cultured on MS (1962) mineral nutrient medium supplemented with 0.62 mg/l–5.0 mg/l of 2,4-D, was established. Initially various types of callus were observed and a correlation between genotype, size of explant and 2,4-D concentration was found. The best embryogenic response was observed when explants were smaller than 1.0 mm. Induction of somatic embryogenesis of 2.0 mm–3.0 mm explants required a higher concentration of 2,4-D. Most embryoids were formed in the presence of 5.0 mg/l of 2,4-D. *Secale africanum* and *S. kuprianovii* appeared to have the highest embryogenic capacity among the five investigated species. For embryoids germination to plantlets the MS medium supplemented with GA_3 and cytokinins was used. Ultimately, out of the 932 regenerants obtained 364 originated from somatic embryogenesis.

Key words: Wild species of *Secale* genus – Somatic embryogenesis – Organogenesis – Regenerants – 2,4-D

Introduction

During the last few years considerable progress in the tissue culture of monocot plants with special attention paid to somatic embryogenesis has been made. Until now only common rye, because of its agronomic importance in Europe, has been chosen for the study of the morphogenic potential of the *Secale* genus.

For this purpose an immature embryo or its fragments appeared to be the best experimental material (Rybczyński 1979; Eapon and Rao 1982; Krumbigel et al. 1984; Lu et al. 1984; Rybczyński and Zimny 1985) although immature inflorescences and rachis of *S. cereale* were also recognized to be capable of plant regeneration (Rybczyński et al. 1980; Zimny and Lörz 1985). Recently, using dicamba as the factor stimulating somatic embryogenesis and plantlet regeneration from meristematic explants of common rye, seedlings and inflorescences have been described (Zimny and Lörz 1985).

The present study is the first attempt to describe the process of somatic embryogenesis and organogenesis of five selected wild species of the *Secale* genus.

Material and methods

Immature embryos of five wild species of the *Secale* genus, i.e., *S. africanum, S. ancestrale, S. kuprianovii, S. segetale* and *S. vavilovii,* which are included in the collection of the Rye Genes Bank in the Botanical Garden of the Polish Academy of Sciences, were dissected from kernels of field grown plants. Methods of plant material sterilization and embryo isolation have already been described (Rybczyński and Zimny 1985). A total of 2,755 embryos were used (Table 1). According to the maturity of the explants three sizes were chosen: smaller than 1.0 mm, 1.0–2.0 mm and larger than 2.0 mm. The culture was established on MS (1962) nutrient medium supplemented with 0.62, 1.25, 2.50, 5.0 mg/l of 2,4-D. The pH of the medium was adjusted to 5.6. The medium was solidified with 7.0 g/l of Difco-Bacto agar. Cultures were held in Petri dishes sealed with parafilm or in 100 ml conical flasks and placed in a culture chamber at 22° ± 1 °C in darkness or in light with a photoperiod of 16/8 h day/night.

Subsequent cultures were maintained on MS (1962) basal nutrient medium supplemented with 0.5–2.0 mg/l of 2,4-D and 500 mg/l of yeast extract or/and 200 mg/l and 750 mg/l of casein hydrolizate.

In order to induce the development of somatic embryos and embryoidal structures to plantlets the following combina-

Abbreviations: 2,4-D = 2,4-dichlorophenoxyacetic acid; GA3 = Gibberellic acid; BAP = Benzylaminopurine

Table 1. Numbers of immature embryos of five wild *Secale* species used for culture on MS medium supplemented with 2,4-D

Species	Type of development	No. of explants					
		mg/l					
		Control	0.62	1.25	2.50	5.00	Total no.
S. africanum (Stapf)	perennial	–	145	144	191	220	700
S. ancestrale (Zhuk)	annual	–	149	145	77	175	546
S. kuprianovii (Grossh)	perential	–	125	117	102	125	469
S. segetale (Rosher)	annual	146	152	152	118	143	711
S. vavilovii (Grossh)	annual	20	26	0	164	119	329

Table 2. The effect of both 2,4-D concentration and size of the explant on embryogenic callus formation of five wild *Secale* species after 2 weeks of the culture

Species	Embryogenic callus formation (% of placed explants)											
	MS (1962) medium + 2,4-D mg/l											
	0.62			1.25			2.50			5.00		
	Size of explant (mm)											
	1	1–2	2–3	1	1–2	2–3	1	1–2	2–3	1	1–2	2–3
S. africanum	19.7	15.2	0	19.1	18.2	3.2	44.0	17.7	0	30.3	23.7	10.3
S. ancestrale	18.4	1.0	–	1.3	2.8	–	3.7	20.0	–	7.0	34.0	8.0
S. kuprianovii	24.0	3.0	–	13.5	9.3	–	22.2	9.6	–	20.0	6.0	–
S. segetale	17.3	10.3	9.4	21.9	13.5	14.7	28.1	7.7	15.6	43.7	49.1	11.5
S. vavilovii	–	0	–	–	–	–	–	6.5	3.0	–	8.5	0

tions of growth substances were used: 1.0 mg/l of Zeatin, kinetin or BAP with 0.1 mg/l GA_3, and 0.1 mg/l GA_3 alone. On the other hand, regenerants obtained by organogenesis were transferred only to the MS medium without growth substances. All regenerants were transferred once more to the 0.5 MS medium and stored in 200 ml conical flasks in the cold room at 10 °C and with a photoperiod of 10/14 h day/night with low intensity light.

Results

1 Preliminary response and types of callus

After two weeks of culture, various primary explant responses were observed depending on the size of the explants (stage of development), genotype and 2,4-D concentration (Table 2). In the control cultures all explants germinated independently of their stage. The increase of 2,4-D in the medium inhibited the development of the embryo axis and affected the overgrowth of the scutellum. This effect was most visible in explants smaller than 1.0 mm and then in 1.0–2.0 mm explants. Two-three mm rye embryo responded very similarily, but the number of germinating embryos was the highest for the investigated species and estimated to be 50% of the used explants.

In our cultures various types of callus were achieved. In a positive correlation with an increase of the 2,4-D concentration in the medium, the number of explants formed friable callus which after two or four weeks of the culture formed numerous centers of nodular callus which appeared to be the source of somatic embryo formation. The nodular callus was directly formed by the scutellum of the explant cultured on the initial medium. The initiation of this type of callus was connected to the margin of the scutellum. For example, in the case of *S. africanum* the nodular structures which were formed by the scutellum developed directly to embryoids missing the callus tissue stage. Only a few explants of all the species investigated resulted in gel-like callus consisting of separately living cells (Fig. 2), and subcultures of this type of callus was unsuccessful.

2 Effect of 2,4-D concentration on embryogenic callus formation

Together with the increase of 2,4-D in the medium the number of the five species' explants investigated forming embryogenic callus increased (Table 2). Among the five investigated species the highest embryogenic

response was visible in two species – *S. africanum* and *S. segetale.* Embryogenic callus, however, was formed at all investigated stages of the explants. In the case of *S. kuprianovii,* the best embryogenic response was seen in the smallest explants with no correlation to 2,4-D concentration. At 2.5 and 5.0 mg/l of 2,4-D the 1.0–2.0 mm long *S. ancestrale* explants appeared to form embryogenic callus with a frequency of 20% and 30%, respectively.

3 Somatic embryogenesis

A considerable variability in somatic embryo formation and plantlet regeneration was species dependent. After 6–8 weeks of the culture of the explants the first very well organized somatic embryos of all investigated species were found in various intensities. The best embryogenic callus was formed by explants smaller than 1.0 mm (Figs. 3 and 4). The highest number of embryoids was achieved in the case of *S. africanum* explant culture in the presence of 1.25–5.0 mg/l 2,4-D. Together with the increase in 2,4-D concentration the number of formed embryoids increased. A higher concentration of 2,4-D simultaneously stimulated somatic embryogenesis in older explants of *S. kuprianovii* and *S. segetale.* Only in the case of *S. kuprianovii* and *S. africanum* was the process of somatic embryogenesis maintained in long term culture in the presence of 2,4-D and 750 mg/l casein hydrolizate. Regenerated embryoids retained a dipolar structure of the monocot zygotic embryo (Fig. 5) but numerous developmental disturbances were observed (Fig. 6).

After three days of culture on germinating medium, embryoids started to form plantlets and, in the case of cultures placed on the medium supplemented with GA_3 and kinetin, the number of embryoid derived plantlets was the highest (Figs. 7 and 8) Table 3 summerizes the final results by presenting the number of plantlets obtained by somatic embryogenesis and organogenesis.

4 Organogenesis

Apart from somatic embryogenesis the phenomenon of plant differentiation by organogenesis was observed. Numerous coleoptiles and leaf primordia were more frequently visible on the surface of friable callus. A higher concentration of 2,4-D (2.5 and 5.0 mg/l) stimulated the formation of coleoptiles and shoot primordia regardless of explant size; a lower concentration (0.62 and 1.25 mg/l) brought about shoot induction at a higher level with the smallest explants. Only a few regenerants via organogenesis of *S. africanum* and *S. ancestrale* were ascertained in the case of the older explants culture.

Discussion

Table 3 clearly shows large interspecies differences in the capacities to form somatic embryos. The first group of investigated wild species of rye is formed by perennials – *S. africanum* and *S. kuprianovii* – and is characterized by a high embryogenic response with respect to the number of embryoids formed; the second group is composed of annual species – *S. ancestrale, S. segetale* and *S. vavilovii* – which has a lower tendency to create somatic embryos.

The effect of 2,4-D, which possesses an phenoxy active group, resulted in the inhibition of axis development of explant together with an overgrowth and swelling of its scutellum. However, the embryogenic response of embryos of investigated species was dependent on maturity of the explant, species and concentration of auxin.

The best embryogenic response was observed with explants smaller than 1.0 mm. Induction of somatic embryogenesis of the oldest explants required a higher concentration of 2,4-D. It has been previously reported that in the case of *S. cereale* a significant decrease in

Table 3. Correlation between the embryogenic response of culture explants and number of regenerants obtained via somatic embryogenesis and organogenesis of the five investigated wild species of *Secale genus*

Species	Total no. of cultured explants	% of explants with embryogenic response after 2 weeks of culture	No. of regenerants		
			Somatic embryogenesis		Organogenesis regenerants
			embryoids	/ regenerants	
S. africanum	700	20.0	285	/ 160	196
S. ancestrale	546	9.2	10	/ 5	200
S. kuprianovii	489	9.2	236	/ 133	33
S. segetale	711	15.5	40	/ 36	116
S. vavilovii	329	2,7	30	/ 30	23

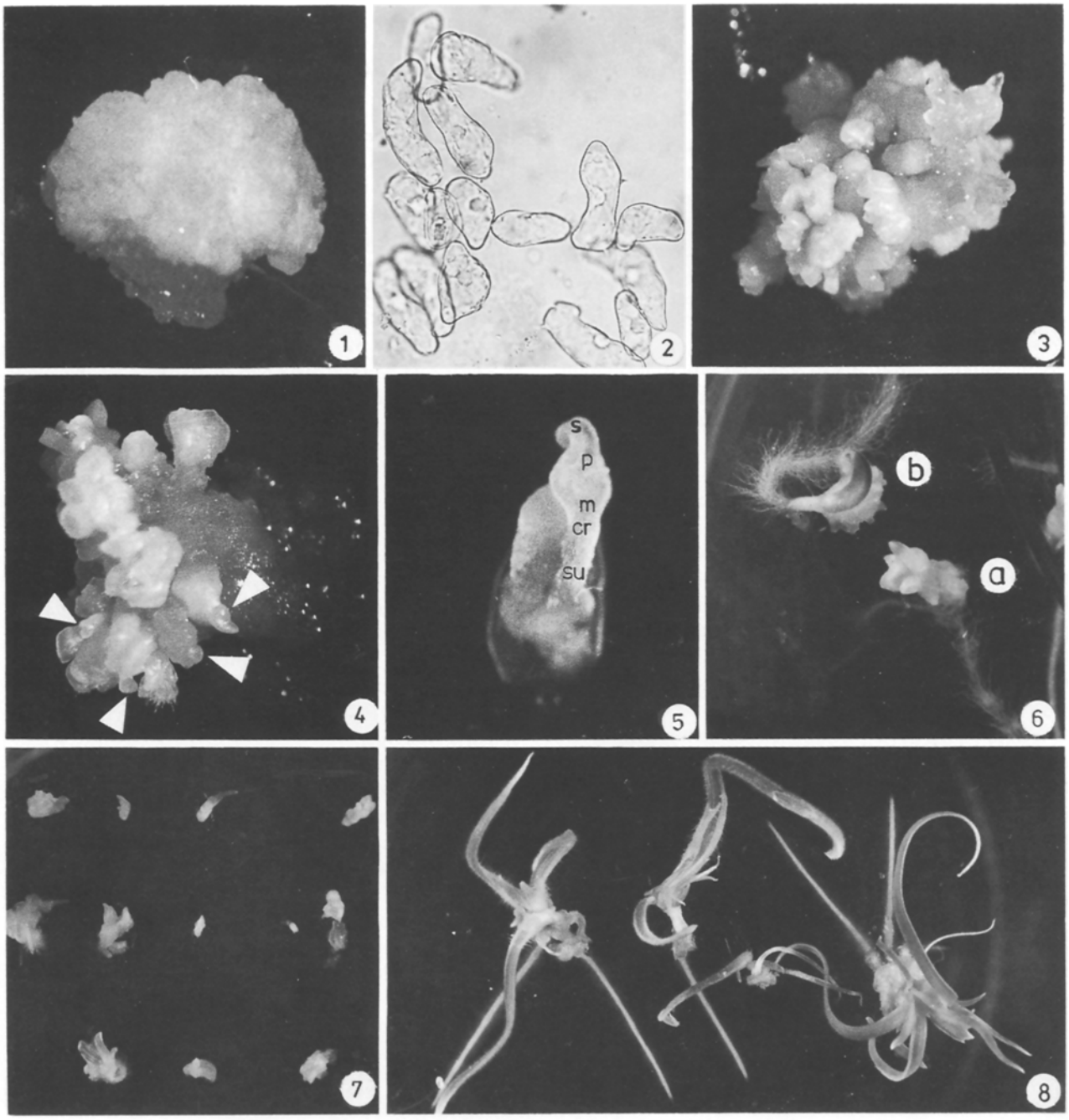

Fig. 1. The inhibition of 1–2 mm long *S. africanum* embryo axis and swelling of its scutellum on MS medium supplemented with 2.5 mg/l of 2,4-D

Fig. 2. Single cells of gelling callus of *S. aftricanum* obtained on MS medium supplemented with 1.25 mg/l 2,4-D

Fig. 3. Numerous embryoidal structures of *S. africanum* induced by 5.0 mg/l of 2,4-D on explants smaller than 1.0 mm

Fig. 4. Various stages of *S. africanum* somatic embryos (*arrowheads*)

Fig. 5. Dipolar, completely regenerated embryo of *S. segetale* with scutellum – s, plumula – p, mesocotyl – m, coleorhiza – cr and suspensor – su

Fig. 6. Two types of somatic embryogenesis disturbances: **a** Twin embryoids with blended scutellum; **b** Embroid with large scutellum possessing numerous transparent plumules

Fig. 7. Three-day-old culture of *S. segetale* somatic embryos germinated on MS medium supplemented with 0.1 mg/l of GA_3 and 1.0 mg/l of kinetin

Fig. 8. Embryoid derived regenerants ready for subculture on hormone free MS medium

the embryogenic response was observed with an increase of 2,4-D from 9.0 μM to 36 μM for meristematic explants of seedling and inflorescences (Zimny and Lörz 1985) with an optimal concentration of 2,4-D 2.5 mg/l in the presence of 3–6% sucrose for the immature embryo (Lu et al. 1984).

Close observation of the early stages of culture allowed us to distinguish various types of callus from single cells constituted gelling tissue to embryogenic tissue. Gelling callus did not survive the subculture though its single-cell constitution might be a reason for initiating attempts to induce its cell division. Watery and gelatinous callus of *Dactylis glomerata*, having the highest tendency to become embryogenic, has already been described (Hanning and Conger 1982).

Even though the initial embryogenic response of *S. ancestrale* and *S. segetale* explants was estimated to be 32% and 49%, respectively, the final number of regenerants obtained via somatic embryogenesis was lower than in the cultures of *S. africanum* and *S. kuprianovii*.

Only in long term cultures of embryogenic callus of *S. africanum* and *S. kuprianovii* subcultured on the medium supplemented with 2,4-D and casein hydrolizate were successive stages of somatic embryo development observed.

Using media containing GA_3 and cytokinins for the further development of embryoids, high percentages of regenerants obtained via somatic embryogenesis were achieved. Various capacities for germination could be dependent on somatic embryogenesis disturbances observed in carried out cultures and stage of embryoidogenesis of placed somatic embryos.

In conclusion we were able to develop the culture system for plant regeneration via somatic embryogenesis of five selected wild species of *Secale* genus and maintain the culture of embryogenic callus of *S. africanum* and *S. kuprianovii* for a long time. Consequently, the next wild species of *Secale* genus will be studied in order to induce somatic embryogenesis.

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