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# SOMATIC EMBRYOGENESIS AND PLANT REGENERATION FROM EMBRYO RESCUE CULTURES OF $F_1$ HYBRIDS OF Teff WITH ITS WILD RELATIVES

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## ABSTRACT

The application of tissue culture techniques, particularly in the area of embryo rescue, has had a major impact on the maintenance and development of hybrid embryo from wide crosses. Embryo rescue techniques are directed towards obtaining more efficient survival of embryos in situations where very immature embryos are to be cultured. An *in vitro* embryo rescue culture technique has been developed for $F_1$ hybridsof *Eragrostis tef* with its wild relativesin order to transgress desirable characteristics to this species that may alleviate the shortcomings of conventional breeding. Eight $F_1$ hybrids were taken from the cross of two wild species of *E. tef*; namely *E. pilosa* accessions30-5 and 37 80 82; and *E. curvula* with 4 cultivars of *E. tef*; namely Kaye Murri, DZ-Cr-387, DZ-Cr-37 and DZ-01-196. Florets were excised from panicles after six days of artificial crossing, and cultured in Murashige and Skoog (MS) medium supplemented with 6 different concentrations of 2,4-D. Among these concentrations, 4.5 µM and 0.45 µM 2,4-D were found to be the best for induction of somatic embryos from the cross of *E. pilosa* (30-5)X'Kaye Murri' (46.7%) and *E. pilosa* (30-5)X'DZ-Cr-37' (13.3%), respectively. Twenty one somatic embryos were obtained from a total of 635 cultured florets of $F_1$ hybrids. Somatic embryos were regenerated into plantlets in half MS medium without plant growth regulators. Plantlets of $F_1$ hybrids of *E. pilosa* (30-5)X'Kaye Murri' were successfully transferred into pots. A total of 442 single plantlets were regenerated till maturity. They were uniform, normal, fertile and had no abberations. Morphologically, the hybrid plantlets showed inherited characteristics of both parents.

*Key Words*: *Eragrostis Tef*, florets, hybrids, wild variety

## RÉSUMÉ

L'application des techniques de la culture des tissues, particulièrement dans le cadre de la préservation de l'embryon, avait eu un impact majeur sur le maintien et le développement d'embryon hybrides dans beaucoup de croisements. Les techniques de préservation embryonnaire sont orientées vers l'obtention de la survie plus efficace d'embryons dans des situations où des embryons très immatures doivent être cultivés. Une technique de culture in vitro pour la préservation embryonnaire a été développée pour $F_1$ *hybrids of Eragrostis tef* avecses homologues sauvages afin de transgresser les caractéristiquesdésirablesaux espèces qui pourraient pallier aux défauts résultant de l'amélioration conventionnelle. Huit hybrides $F_1$ issus du croisement de deux espèces sauvages de *E. tef*; nommément, les accessions *E. pilosa* 30-5 et 37 80 82; et *E. curvula*avec 4 cultivars de *E. tef*; nommément Kaye Murri, DZ-Cr-387, DZ-Cr-37 et DZ-01-196. Apres six jours de croisement artificiel, les fleurs étaientexcisées et cultivées dans les media de Murashige et Skoog (MS) avec un supplément de 6 concentrations différentes de 2,4-D. Parmi ces concentrations, 4.5 µM et 0.45 µM 2,4-D étaienttrouvées les meilleures pour l'induction d'embryons somatiques de croisement de *E. pilosa* (30-5)X'Kaye Murri' (46.7%) et *E. pilosa* (30-5)X'DZ-Cr-37' (13.3%), respectivement. Vingt et un embryons somatiques étaient obtenus du total de 635 cultures de fleurs des hybrids $F_1$. Les embryonssomatiques étaient régénérés en plantules dans la

moitié du medium MS sans régulateurs de la croissance de plants. Les plantules d'hybrides F1 de *E. pilosa* (30-5) X'Kaye Murri' étaient transferees avec succès dans des pots.Un total de 442 plantules simples était régénéré jusqu'à la maturité. Elles étaient uniformes, normales, fertileset n'avaient aucun défaut. Morphologiquement, les plantules hybrides ont montré des caractéristiques héritées des deux parents.

*Mots Clés*: *Eragrostis Tef,* fleurs, hybrides, variétés sauvages

## INTRODUCTION

*Eragrostis tef* (Zucc.) Trotter commonly known as teff belongs to class: Liliopsida, order: Poale, family: Poaceae, subfamily: Chloridodeae, genus: *Eragrostis* (Smale *et al*., 1996). Teff is a fine stemmed tufted annual grass (Stallknecht *et al*., 1993) which is predominantly selfing, a $C_4$ and annual crop plants (Waines and Hegde, 2003).Teff is originated, dominantly cultivated, and a major staple food crop in Ethiopia. It is perhaps descended from the closely related wild *Eragrostis pilosa* (L.) P. Beauv (Ingram and Doyle, 2003).

Teff is a good source of minerals, particularly calcium and iron to human (Mengesha *et al*., 1965). According to the report of Areda (1995), teff grain protein was in the average range of 11.2-12.5 percent. Moreover, cattleprefer teff straw over straw of other cereals, and its quality is comparable to good natural pasture (Ketema, 1997).

Some of the production constraints of teff are late maturation, tedious crossing techniques, weak floral organ, lodging, unique pollination habit, small size of the floral parts, insect pests, fungal diseases and others(Stallknecht *et al*., 1993; Ketema, 1997; Gugsa, 2005; Yu *et al*., 2007). Except few studies, teff has not been studied using modern tools of biotechnology (Yifru and Tefera, 2005).

The occurrence of post fertilisation disorders constitutes a major hurdle to stable hybrid embryo development in wide crosses through conventional breeding. Implementation of molecular and cellular genetic methods in a breeding programme requires an efficient regeneration system from somatic embryogenesis.

The application of tissue culture techniques, particularly in the area of embryo rescue, has had a major impact on the maintenance and development of hybrid embryo from wide crosses. Embryo rescue techniques are directed towards obtaining more efficient survival of embryos in situations where very immature embryos are to be cultured (Niimi *et al*., 1995; Raghavan, 2003).

Somatic embryos in teff have been developed from roots and leaves (Bekele, 1985), mature seeds (Assefa *et al*., 1998) and immature embryos (Gugsa *et al*., 2006); pistle (Gugsa, 2005). However, somatic embryos from $F_1$ hybrids of teff either among domesticated varieties or with its wild relatives have not been achieved so far.

The objective of thisstudy was to regenerate $F_1$ hybrid plants, optimise the developmental stages of hybrid florets for the induction of somatic embryos, and optimise the concentrations of 2,4-D to induce somatic embryos from interspecific crosses of *Eragrostis tef* with its wild relatives.

## MATERIALS AND METHODS

**Plant materials.** The seeds of two wild species of *Eragrostis*: *Eragrostis pilosa* (30-5), *Eragrostis pilosa* (37 80 82) and *Eragrostis curvula,* and four varieties of teff: namely Kaye Murri, DZ-Cr-387, DZ-Cr-37 and DZ-01-196 were used in this study. They were obtained from teff sections of Debre Zeit Agricultural Research Center (DZARC) in Ethiopia. The seeds were sown in 90 mm diameter Petri-dishes until they were germinated. After 8-10 days, they were transplanted and grown in small sized pots of 11-13 cm diameter, containing light soils in the glasshouse. The seeds of both accessions of *E. pilosa* were sown one week later and that of *E. curvula* a week earlier than the sowing date of teff varieties.

**Interspecific crossing of *Eragrostis tef* with its wild relatives.** At the heading stage, the anthers of wild species were emasculated in the afternoon (3.00-4.00 pm) under a stereo-microscope, using very fine forceps and needles. To avoid confusion

of identifying pollinated and unpollinated florets of the mother plants, all the florets around the emasculated florets were removed. The wild species and domesticated varieties were used as female and male plants, respectively. The domesticated varieties were kept in a refrigerator overnight. The next day, from 6.00-9.00 am, stamens were excised from the male plant, and pollen was transferred to the female plant. Eight cross combinations were carried out. These were;the cross combination of *E. pilosa* (30-5)with DZ-Cr-37, DZ-Cr-387, DZ-01-196 and Kaye Murri; *E. pilosa* (378082) with DZ-Cr-37, DZ-Cr-387, and Kaye Murri; and *E. curvula* with DZ-Cr-387. Pollinated florets from the eight cross combinations were excised after 3-12 days of artificial crossing and cultured on induction mediumsupplemented with different concentrations of 2,4-D (0, 0.45, 2.25, 4.5, 6.75, 9.0 µM).

**Collection of panicles and surface sterilisation.** After 3-12 days of artificial crossing, panicles were removed from the plants and the spikelets were cut using sterilised scissors, scalpel and forceps. They were sterilised with 4% sodium hypochlorite of the original 5.25% (w/v) for eight minutes, and rinsed four times with sterile double distilled water.

**Excision of florets, pollinated ovaries and somatic embryos.** The florets from sterilised spikelets were detached and cultured in 100 mm x15 mm Petri-dishes containing 20 ml MS medium, supplemented with 0, 0.45, 2.25, 1, 6.75, and 9.0 µM2,4-D; sealed with parafilm and kept in light culture condition. During the first subculturing, ovaries were excised from the cultured florets and transferred into the same induction medium. Somatic embryos were excised under stereo-microscopy from the cultured pollinated ovaries; and they were transferred into regeneration medium.

**Harvesting stages of panicles for induction of somatic embryos.** $F_1$ hybrids of *E. pilosa* (30-5) x *E. tef* cv. Kaye Murri were used to determine appropriate harvesting stages of panicles for induction of better percentage of somatic embryos. After artificial crossing, panicles were collected for different periods (3, 6, 9 and 12 days). Their florets were excised and placed in petri-dishes containing 20 ml MS medium supplemented with 4.5 µM of 2,4-D and 30 g $l^{-1}$ maltose. The Petri dishes were placed under light culture condition.

**2,4-D concentrations *versus* induction of somatic embryos.** After 5-6 days of artificial crossing, pollinated florets of $F_1$ hybrids of teff with its wild relatives were cultured in MS medium, supplemented with 0, 0.45, 2.25, 4.5, 6.75, and 9.0 µM2,4-D and 30 g $l^{-1}$ of maltose. They were kept in light culture condition. After 5-6 weeks from the initial culture, somatic embryos were transferred into Petri-dishes containing 20 ml of regeneration medium. The regeneration medium was half strength of MS medium, without growth regulator. The first subculturing of the embryos was done in the same fresh medium of the regeneration medium. The second subculturing of the embryos was done in test tubes contanining 15 ml of the regeneration medium. Finally, they were subcultured in Majenta jars containing 50 ml of the regeneration medium.

**Acclimatisation.** Plantlets of teff $F_1$ hybrids from the cross of *Pilosa* (30-5) and *tef* cv. Kaye Murri were removed from Majenta jar using forceps. The roots were washed with distilled water to remove the gelrite. They were placed into pots containing with 3:2:1 ratios of black soil, compost and sand, respectively. Each pot was covered with a plastic bag for two weeks, and placed in the growthroom. The plastic bags were half removed after one week. After two weeks, the pots were transferred into glasshouse. Morphological characteristics such as plant height, panicle length, culm length and peduncle length of rescued hybrids of the main tiller were measured and the total number of tillers were counted from each pot.

**Data collections and analysis.** Each independent experiment had 1-3 replications and 1-4 repeats. In this study, 5 pollinated florets and 2-3 somatic embryos were used as a unit of replication to induction and regeneration medium, respectively. That wasbecause the probability of gettingsuccessful hybrid florets was very limited. Subculturing was carried out every 2-3 weeks.

The responses of cultured florets were checked every week and the frequency of induced somatic embryos were recorded per Petri dish for each treatment.

All the experimentswere carried out in complete random design. The data were subjected to analysis of variance (ANOVA) using SAScomputer software (Version, 1999). The possible pairs of treatment means werecompared using LSD test at $P \leq 0.05$.

## RESULTS

**Developmental stage of immature embryos of $F_1$ hybrids.** The effect of different developmental stages of immature embryos on the percentage of induced somatic embryos indicated that florets cultured 6 days after artificial crossing produced the maximum somatic embryo (60%), followed by 3 and 9 days that produced the same frequency (20%) of somatic embryos (Table 1).

**2,4-D versus induction of somatic embryos.** The effect of different concentrations of 2,4-D on induction of somatic embryos is presented in Table 2. It is clear that the $F_1$ hybrids of *E. pilosa* (30-5) x Kaye Murri induced maximum percentage of somatic embryos in 4.5 µM 2,4-D (46.7%). The $F_1$ hybrids of *E. pilosa* (30-5) x DZ-Cr-37 induced somatic embryos only in 0.45 µM 2,4-D (13.3%).However, the $F_1$ hybrids of *E. pilosa* (30-5) x DZ-01-196, *E. pilosa* (30-5) x DZ-Cr-387, *E. pilosa* (37 80 87) x Kaye Murri, *E. pilosa* (37 80 87) x DZ-Cr-387, *E. pilosa* (37 80 87) x DZ-Cr-37, and *E. curvula* x DZ-Cr-387 did not induce somatic embryos in any of the 2,4-D concentrations.

Overall, there were significant differences among treatments and parental combinations indepedently on induction of somatic embryos (Table 3). The parental combination of *E. Pilosa* (30-5) x Kaye Murri was significantly different from the other seven parental combinations. The remaining seven parental combinations were not significantly different among themselves.

**Regeneration of plantlets from somatic embryos.** After 5-6 weeks, from a total of 635 cultured florets of $F_1$ hybrids, 21 somatic embryos (3.3%) of $F_1$ hybrids were obtained. Of these, 19 somatic embryos were from the cross of *E. pilosa* (30-5) with Kaye Murri, and 2 somatic embryos were from the cross of *E. pilosa* (30-5) with DZ-Cr-37. Somatic embryos were transferred into regeneration medium, half strength MS medium without growth regulator supplemented with 30 g $l^{-1}$ of maltose. Embryos were differentaited into 1-5 shoots per explant. During the third subculturing, shoots were subcultured in the same medium and induced numerous roots. Nineteen somatic embryos of *E. pilosa* (30-5) x Kaye Murri, 14 somatic embryos (73.7 %) were differentaited into shoots. A single somatic embryo could give 1-10 plantlets. From two somatic embryos of *E. pilosa* (30-5) x DZ-Cr-37, only 1 shoot was regenerated.

**Acclimatisation.** All plantlets survived in the growthroom and were transferred into glasshouse. In the glasshouse, after three days, only three plantlets died due to fungal contamination in the growthroom. They were grown to maturity in the same glasshouse. The $F_1$ hybrids shared qualitative and quantitative characteristics from both parents. For instance, panicle colour, seed colour, vasal stock colour and other characteristics of the hybrids have been derived from Kaye Murri. Panicle form, peduncle length, height and other characteristics

TABLE 1. The effect of developmental stage of immature embryos on induction of somatic embryo (%) using $F_1$ hybrid of *E. pilosa* (30-5)*Kaye Murri

| Days after artificial crossing (daac) | CF | SE | % SE |
|---|---|---|---|
| 3 | 5 | 1 | 20.0 |
| 6 | 5 | 3 | 60.0 |
| 9 | 5 | 1 | 20.0 |
| 12 | 5 | 0 | 0.0 |

daac = days after artificial crossing, CF = cultured florets, SE = somatic embryo

TABLE 2. Effect of 2,4-D on induction of somatic embryos. Mean values are shown as ± SD

| Parental combination | Treatments | | | | | |
|---|---|---|---|---|---|---|
| | 0 µM 2,4-D | 0.45 µM 2,4-D | 2.25 µM 2,4-D | 4.5 µM 2,4-D | 6.75 µM 2,4-D | 9.0 µM 2,4-D |
| *E. pilosa* (30-5)* Kaye Murri | 0.0±0.0a | 13..3±5.5b | 20.0±0.0b | 46.7±9.4c | 13.3±5.5b | 0.0±0.0a |
| *E. pilosa* (30-5)*DZ-Cr-37 | 0.0±0.0a | 13.3±5.5b | 0.0±0.0a | 0.0±0.0a | 0.0±0.0a | 0.0±0.0a |
| *E. pilosa* (30-5)*DZ-01-196 | 0.0±0.0a | 0.0±0.0a | 0.0±0.0a | 0.0±0.0a | 0.0±0.0a | 0.0±0.0a |
| *E. pilosa* (30-5)*DZ-Cr-387 | 0.0±0.0a | - | - | 0.0±0.0a | 0.0±0.0a | - |
| *E. pilosa* (37 80 87)*Kaye Murri | 0.0±0.0a | 0.0±0.0a | 0.0±0.0a | 0.0±0.0a | 0.0±0.0a | 0.0±0.0a |
| *E. pilosa* (37 80 87)* DZ-Cr-37 | 0.0±0.0a | 0.0±0.0a | 0.0±0.0a | 0.0±0.0a | 0.0±0.0a | - |
| *E. pilosa* (37 80 87)* DZ-Cr-387 | 0.0±0.0a | 0.0±0.0a | 0.0±0.0a | 0.0±0.0a | 0.0±0.0a | 0.0±0.0a |
| *E. curvula*DZ-Cr- 387 | - | - | 0.0±0.0a | 0.0±0.0a | - | 0.0±0.0a |

Similar letters along the rows or columns indicated that there is no significant difference at P≤0.05

have been derived from *E. pilosa* (30-5). At maturity, each plantlet in a pot induced 8-40 tillers, all pots together induced a total of 442 single plantlets. All plantlets of the $F_1$ hybrid were green and fertile. Some quantitative characteristics of the main tillers were evaluated. According to the results, the average plant height, panicle length, peduncle length and culm length were 93.3±12.1, 30.2±3.4, 21.9±5.5 and 63.2±9.8, respectively.

## DISCUSSION

**Developmental stage of immature embryos.** Developmental stages of immature embryos indicated that out of the four developmental stages (3, 6, 9 and 12 days after interspecific crossing (daac)), 6 days was the optimum developmental stage for induction of somatic embryos. This is in agreement with immature pollinated pistilate cultures of teff (Gugsa, 2005). This best developmental stage was accompanied with excision of immature zygotic embryos from the pollinated florets with stereo microscope. In 3 and 9 days, there was small percentages (20%) of somatic embryo formation; however, the 12 days did not set immature zygotic embryo at all. At the early stages of development, there was very low chance of seed setting or immature zygotic embryo formation and as the number of days increases after artificial crossing, there might have been a high chance of embryo abortion due to loss of functionality of gametes. In immature zygotic embryo cultures of teff, 10-12 days after pollination were the optimum (Gugsa, 2005). The types of explants taken during the first culture might be the possible reason for the differences.

**2,4-D and induction of somatic embryo.** In the present study, somatic embryos were induced *via* direct somatic embryogenesis using 0-9.0 µM 2,4-D, which reduced the time required for plant regeneration, beneficial to minimise culture-induced genetic changes and avoid the formation of somaclonal variations. Eight interspecific crosses between domesticated and wild relatives of teff were carried out and their florets were cultured in six different 2,4-D concentrations. Somatic embryos were induced only from the floret cultures of two $F_1$ hybrids. This might be

TABLE 3. Analysis of variance for the effect of parental combinations and treatments on induction of somatic embryos

| Source of variation | DF | MS | F-value | P |
|---|---|---|---|---|
| Parental combination | 7 | 533..3 | 12.83 | <0.0001** |
| Treatment | 5 | 117.8 | 2.83 | 0.0184* |
| Rep | 2 | 2.8 | 0.07 | 0.94 |
| Error | 129 | 41.6 | | |
| Total | 143 | 695.5 | | |

*= significant difference at P≤0.05, **= significant difference at P≤0.01

due to incomptability of gamates, abortion of embryos, and damaging of immature zygotic embryos during mechanical manipulation at very early and late stages of development. Floret cultures from $F_1$ hybrids of *E. pilosa* (30-5) x Kaye Murri induced somatic embryos in all 2,4-D concentrations, except in the control and 9.0 µM 2,4-D. The absence of 2,4-D, and presence of high concentration of 2,4-D, reduced the fromation of somatic embryos. However, floret cultures of $F_1$ hybrids of *E. pilosa* (30-5) x DZ-Cr-37 induced somatic embryos only in 0.45 µM 2,4-D. The florate cultures of $F_1$ hybrids of *E. pilosa* (30-5) x Kaye Murri induced the highest (46.7%) somatic embryos in 4.5 µM 2,4-D, followed by 2.25 µM 2,4-D (20%). The highest percentage of somatic embryos was induced in a 2,4-D concentration, within the range of auxins (1-5 mg $l^{-1}$ dicamba) used in the work of Mekbib *et al.* (1997). This study was also in agreement with the induction of calli from immature zygotic embryo cultures of bread wheat. Maximum calli were induced in 4.5 µM 2,4-D (Sears and Deckard, 1982).

From a total of 635 cultured pollinated florets of $F_1$ hybrids, 21 somatic embryos (3.3%) of $F_1$ hybrids were obtained, and depending on the parental combinations and treatment conditions, 0-46.7% somatic embryos were observed. The present study is the first in its kind to achieve high regeneration from $F_1$ explant of teff and its wild relatives. This finding is in agreement with ovule cultures of barely (Töpfer and Steinbiss, 1985) and mature seed cultures of teff (Assefa *et al.*, 1998).

In this study of embryo rescue technique, it was shown that allplantlets were fertile from the crosses where the female parent was the wild species.The achievement of seed settings from the cross of *E. pilosa* (30-5) x DZ-Cr-37 and *E. pilosa* (30-5) x Kaye Murri was the first success in crossing of teff. In tissue culture works, the achievement of somatic embryos from $F_1$ hybrids of teff from both crosses was the first work. The study achieved not only fertile progenies but also used to micropropagate the hybrid seeds and reduce the breeding cycles of teff varieties to be released through conventional breeding. However, Gugsa *et al*. (1999) reported that 75% of the $F_1$ hybrids from the cross of tef and *E. pilosa* (30-5) did not set seeds especially when the female was the wild spp. Tefera *et al.* (2003) reported the achievement of interspecific cross between *E. tef* cv. Kaye Murri and *E. pilosa* (30-5) when the wild was served as a pollen donor.

**Regeneration of plantlets from somatic embryos.** During preliminary trials of this study, somatic embryos were transferred into full MS medium without growth regulators, and all somatic embryos died because increased amount of nutrients beyond optimum limit may have inhibited differentiation of the somatic embryos into plantlets. At last, somatic embryos were transferred into half MS medium without growth regulators. Somatic embryos were separated into small pieces and subsequently subcultured in fresh medium, which increased the number of plantlets per somatic embryo. The capacity of somatic embryos to regenerate into plantlets and the high number of plantlets per explant depend on the genotypes, culture media composition and the size of the embryo. In this study, 50-73.5% of the somatic embryos of F1 hybrids of *pilosa* (30-5) x KayeMurri regenerated into shoots. Somatic embryos from the crosses of *E. pilosa* (30-5) x DZ-Cr-37 regenerated one shoot per explant and

*E. pilosa* (30-5) x Kaye Murri regenerated 1-10 shoots per explant.

A similar case was the regeneration of plantlets from capitulum explants of gerbera in half MS medium (Kanwar and Kumar, 2008). Also, somatic embryos of saffron were induced in half MS medium without growth regulator (Sheibani *et al.*, 2007). Subculturing was essential to initiate primary somatic embryo formation for cv. Fesho and secondary somatic embryo formation for cv.Dz-01-196 in somatic embryos culture of teff (Gugsa, 2005).

**Growth conditions of regenerants.** All the regenerants (100%) survived in the growthroom. Most of the regenerants (87.5%) survived after being transferred in the glasshouse, which is very important for future adoption of the technique to facilitate teff improvement. This might be due to potential of teff toresist harsh environmental conditions, which is relatively high compared toother cereals. A few plantlets (12.5%) died in the glasshouse after three days. This may be due to fungal contamination in the growthroom. A similar case was reported by Ayele and Helmut (1995). All plantlets grown in the glasshouse were uniform, normal, fertile and without somaclonal varations. Similar works were reported by Assefa *et al*. (1998) from mature teff seed cultures and from immature zygotic embryo cultures of teff (Gugsa, 2005). As expected for an interspecific cross, distributions of phenotypic values in the progeny showed bi-directional transgressive segregants for all traits, except traits related to lodging which showed transgressive segregants towards only the *E. pilosa* (30-5) parent only. This was in agreement with the results of crossing work of Tefera *et al*. (2003) between *E. tef* and *E. pilosa*. *E. pilosa* (30-5) has contributed useful breeding traits, such as earliness, panicle form, peduncle length, short stature and may be others to the hybrids. Moreover, Kaye Murri may contributed seed colour, vasal stock colour, panicle length, culmlength,and thickerculms and may be other characteristics to the hybrids. At maturity, each somatic embryo induced 8-40 plantlets (tillers) and a total of 442 plantlets (tillers) were produced by the rescued $F_1$ plants.

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