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# Comparison of callus culture with embryo culture at different times of embryo rescue for primary triticale production

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**Summary**

Callus culture of immature wheat-rye hybrid embryos was compared with embryo culture in two experiments. Embryos were rescued from field grown mother plants at two day intervals 13–21 days after pollination and plated for 1) callus culture on Murashige and Skoog medium (MS) supplemented with 2 mg/l 2,4-dichlorophenoxy acetic acid followed by plant regeneration on hormone free MS medium with half strength mineral salts, 2) embryo culture on Taira and Larter medium (TL). Observations were made on embryo size and condition at time of rescue (Experiment 1) and embryo development directly into plants (embryo culture) or through embryogenesis (callus culture). Fewer 19 and 21 day old embryos developed into plants from callus culture than from embryo culture in Experiment 1. Callus culture was more efficient than embryo culture in promoting plant recovery from 17 day old embryos in Experiment 2. The number of plants per embryo was significantly higher from callus culture than from embryo culture. In both experiments callus culture promoted embryogenesis in more embryos than developed in embryo culture. Embryo rescue 15–17 days after pollination was optimal in both experiments.

## Introduction

Genetically diverse populations are important in breeding triticale (× *Triticosecale* Wittmack), a synthetic crop (Varughese et al., 1987). Primary wheat-rye crosses (durum wheat, *Triticum durum* Desf.; *Secale cereale* L.) are valuable for expanding the germplasm base of triticale by exploiting the rye and durum wheat gene pools, which are otherwise difficult to access. The major problem with these crosses is poor endosperm development: embryos abort early in their development. Therefore, embryo rescue and *in vitro* culture are necessary. For crosses done in the greenhouse, embryo rescue at 12–15 days after pollination was recommended (Kaltsikes & Gustafson, 1986).

Callus culture of immature hybrid embryos and subsequent plant regeneration offers an alternative to conventional embryo culture and enables production of several plants from a single embryo. This is important as the majority of amphihaploid plants are lost during colchicine treatment, used to double the chromosome complement (Varughese et al., 1987).

The objectives of this study were to 1) determine the optimal time of embryo rescue in the field and 2) compare callus culture with embryo culture for producing plants from immature wheat-rye hybrid embryos.

## Materials and methods

The crosses between tetraploid wheat and rye were made in the field during two crossing cycles: in Experiment 1, in the central Mexican highlands under rain-fed conditions (19°31′ N 98°50′ W 2249 masl) and in Experiment 2 in the coastal region of northwestern Mexico under irrigated conditions (27°20′ N 109°54′ W 39 masl).

In Experiment 1, the crosses between tetraploid wheats and ryes included three 'easy' ones (observed seed set > 20%): Cocorit 71 × Vigorous Semidwarf (Cross 1), Yavaros 79 × Snoopy Corto/2★Blanco (Cross 2), Sham 3 × Selcurt (Cross 3), and three 'difficult' ones (observed seed set < 6%, no plant recovery): Celta × Harlan J.R. (Cross 4), Erpel/Ruso//Laru × Sardev (Cross 5), Ostrero//Celta/Yavaros 79 × Acconteci (Cross 6). Embryos were excised 13, 15, 17, 19 and 21 days after pollination. Notes were taken on the size and condition of the embryos at the time of rescue. Embryos were divided into three size categories (1, < 0.6 mm long, transparent; 2, ≥ 0.6 mm, < 0.8 mm, transparent-white; 3, ≥ 0.8 mm, white), and three condition categories (1, brown, damaged; 2, granular, very misshapen; 3, normal or slightly misshapen). Experiment 2 comprised four tetraploid wheat × rye crosses: Huitle/Tubeno × Turkey/Sardev (Cross 7), Tancho//Shearwater/Mallard/3/Crex × Turkey/Sardev (Cross 8), Sham 3 × Selcurt/Snoopy (Cross 9) and Yavaros 79 × China/Snoopy (Cross 10). Embryos were excised every two days between 13 and 21 days after pollination. Embryos of different size and condition were divided equally between the two culture media.

Seeds were surface sterilized by washing in 70% ethanol for 1 min, and soaking for 10 min in 20% commercial bleach (6% sodium hypochlorite) and 0.5% polyoxyethylenesorbitan monooleate (Tween 80), followed by five rinses in sterilized water. In both experiments half the embryos were plated on MS medium (Murashige & Skoog, 1962) supplemented with 2 mg/l of 2,4-D (2,4-dichlorophenoxy acetic acid) and 20% sucrose for callus culture, and half on TL medium (Taira & Larter, 1978) for embryo culture. The media were adjusted to pH 5.5 before autoclaving (15 min at + 121° C 20 psi).

For callus culture, the embryos were placed in sterile plastic Petri dishes, two per dish, scutellum side up and the embryo axis embedded in the medium. For embryo culture the embryos were placed in glass vials, two per vial, the embryo axis up and the scutellum side embedded in the medium. Cultures on callus medium were incubated in darkness at + 25° C for five weeks, after which sections of embryogenic calli were subcultured onto hormone free MS medium with half strength mineral salts, for plant regeneration, and cultured at + 21° C in 16 h photoperiod (50 µmol $m^{-2}$ $s^{-1}$, fluorescent light). In Experiment 1 regenerability was checked by subculturing only one embryogenic section per callus. In Experiment 2 all embryogenic callus was subcultured for estimating total plant production. Embryo cultures were kept in + 5° C for two weeks before culture at + 25° C in darkness until shoot emergence. Culture vials were then transferred into light (16 h photoperiod) and cultured at + 21° C.

Frequencies of embryos inducing embryogenic calli and regenerable calli were calculated for callus cultures. Embryogenic callus was recognized by its compact and nodular appearance, pale yellow colour and formation of somatic embryoids. Total plant production was assessed in Experiment 2. Frequencies of germinating embryos were calculated from embryo cultures. Chi-square analyses (INSTAT, 1990) were used to analyse frequency data. Data for total plant production were analysed using a generalised linear model analysis of variance (SAS, 1985). Embryos with no plant production were assinged a value of zero and the data were transformed by $\sqrt{(x+1)}$. 'Least significant difference' was used to separate significantly different means.

## Results

### *Plant recovery in relation to early embryo development and time of rescue*

The proportion of embryos in size category 1 was 54.8% 13 days after pollination and 5.5% 21 days after pollination. However, the size of embryos depended on the cross. Fewer plants were recovered

from small embryos (category 1) than from embryos in categories 2 ($P < 0.001$) and 3 ($P < 0.01$). The condition of the embryos deteriorated significantly from 17 days after pollination. Significantly more plants were recovered from the normal or nearly normal embryos (category 3) than from other embryos ($P < 0.001$).

In Experiment 1, plant recovery from embryos rescued at 13, 15 and 17 days (42.8% of 250, 38.9% of 272 and 37.1% of 271 embryos, respectively) was not significantly different ($P > 0.05$). Plant recovery rate fell significantly with increasing time and was at 19 days 21.3% of 258 embryos and at 21 days 9.5% of 186 embryos. This also applied to large embryos of good condition. Changes in embryo size and condition, and plant recovery by rescue dates in Experiment 1, are shown in Fig. 1. In Experiment 2, significantly fewer embryos developed when rescued at 13 and 21 days than when rescued at other dates ($P < 0.05$). Plant recovery was highest for embryos rescued 15 days after pollination.

Bacterial contamination was seen in cultures begun 21 days after pollination. This seemed to be related to poor embryo condition and watery endosperm.

All the 'easy' crosses and cross 5 (previously classified as 'difficult') in Experiment 1, set seed at >40%. The 'difficult' crosses 4 and 6 set seed at ~10%. Crosses 6 and 1, with the lowest plant recovery, showed slow embryo growth and early embryo deterioration. There was no correlation between seed set and plant recovery from embryos ($R^2 = 0.4674$, df = 1, 4, $F = 3.51$, $P > 0.1342$).

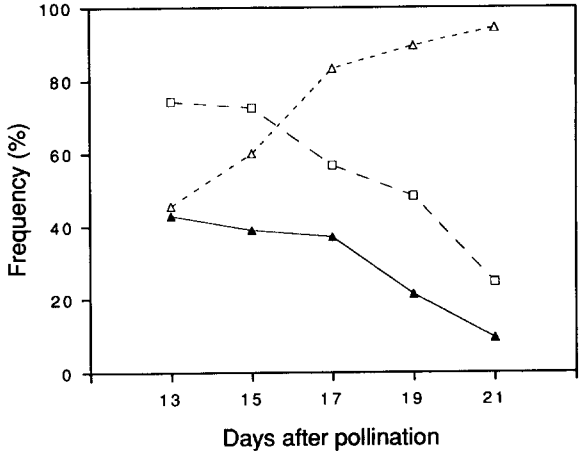


*Fig. 1.* Change in embryo size and condition and respective change in plant recovery from embryos by days after pollination; △—, size categories 2 and 3; □———, condition category 3; ▲---, plant recovery from embryo.

### *Effect of culture method on plant recovery*

Results of induction of embryogenic callus and plant regeneration in callus culture, and embryo germination in embryo culture, are given in Table 1. In Experiment 1, significantly more embryos developed into plants through embryo culture than through callus culture ($P < 0.01$). However, comparing the methods at each rescue date, the differ-

*Table 1.* Development of hybrid wheat-rye embryos through callus culture (production of embryogenic callus, EC, and plant recovery, PL) and embryo culture (PL) for embryos rescues at five dates.

| Medium | Experiment 1 | | | Experiment 2 | | |
|---|---|---|---|---|---|---|
| | MS | | TL PL | MS | | TL PL |
| | EC | PL | | EC | PL | |
| Days[a] | | | | | | |
| 13 | 49.2 (124)b | 37.9 (124)a | 47.6 (126)a | 10.8 (65)b | 7.9 (63)c | 15.1 (53)a |
| 15 | 56.0 (134)ab | 35.4 (127)a | 42.0 (138)a | 40.0 (55)a | 35.8 (53)a | 18.2 (77)a |
| 17 | 61.3 (137)a | 37.6 (125)a | 36.6 (134)ab | 34.2 (76)a | 29.2 (72)a | 16.9 (71)a |
| 19 | 34.6 (133)c | 16.3 (129)b | 26.6 (124)bc | 32.5 (83)a | 21.3 (75)ab | 20.0 (65)a |
| 21 | 15.9 (88)d | 1.3 (79)c | 7.0 (89)c | 13.0 (52)b | 13.5 (52)bc | 9.4 (35)a |

[a] Rescue time, 13, 15, 17, 19, 21 days after pollination. Numbers in parentheses indicate total number of embryos. Numbers in columns with the same letter are not significantly different ($X^2$, $P > 0.05$).

*Table 2.* Development of hybrid wheat-rye embryos through callus culture (production of embryogenic callus, EC, and plant recovery, PL) and embryo culture (PL) for six crosses in Experiment 1 and four crosses in Experiment 2

| Medium | MS | | TL |
|---|---|---|---|
| | EC | PL | PL |
| Experiment 1 | | | |
| Cross 1 | 43.2 (74)a | 25.4 (67)b | 25.9 (68)ab |
| Cross 2 | 36.0 (100)ab | 25.5 (98)b | 43.7 (103)a |
| Cross 3 | 50.0 (112)a | 37.1 (105)a | 43.5 (115)a |
| Cross 4 | 48.3 (118)a | 25.2 (112)b | 36.4 (118)ab |
| Cross 5 | 50.9 (114)a | 29.2 (106)b | 38.8 (116)ab |
| Cross 6 | 41.8 (98)a | 20.8 (96)b | 16.8 (111)b |
| Experiment 2 | | | |
| Cross 7 | 29.2 (65)a | 15.8 (57)ab | 9.4 (64)b |
| Cross 8 | 25.6 (82)a | 22.0 (82)a | 21.0 (81)a |
| Cross 9 | 40.7 (81)a | 34.2 (73)ab | 23.9 (71)b |
| Cross 10 | 15.5 (103)a | 15.7 (103)a | 11.8 (85)a |

Numbers in parentheses indicate the total number of embryos. Numbers in rows with the same letter are not significantly different ($X^2$, $P > 0.05$).

ence in plant recovery between the two culture methods was only significant 19 ($P < 0.05$) and 21 days ($P < 0.001$) after pollination. Significantly more embryos began development on callus medium, and formed embryogenic callus, than on TL medium ($P < 0.001$). Rescue 15 and 17 days after pollination was optimal for embryogenesis (Table 1). However, plant regeneration was only shown by 65.4% of all (246) the embryogenic calli tested for regenerability.

In Experiment 2 significantly more embryos developed into plants through callus culture than through embryo culture ($P > 0.05$). However, comparing the methods at each rescue date, the difference was only significant 17 days after pollination ($P < 0.05$). For callus culture, rescue at 15 and 17 days after pollination was significantly better for plant recovery than rescue at 13 and 21 days ($P < 0.05$). 93.2% of embryogenic calli (73) were regenerable. Similar plant recovery was observed for all rescue dates with embryo culture ($P > 0.05$).

In Experiment 2, callus culture was better for total plant production ($P < 0.001$) compared with embryo culture. Significantly more plants were produced from callus culture than from embryo culture with rescue 15, 17 and 19 days after pollination ($P < 0.05$; Fig. 2).

## Discussion

The optimal time for rescuing wheat-rye hybrid embryos in the greenhouse, to avoid embryo abortion, was suggested to be 12–15 days after pollination (Kaltsikes & Gustafson, 1986). Raina (1984) found that 12–14 day old embryos, rescued from field grown plants, failed to develop, whereas maximum seedling development was obtained with 20–22 day old embryos. The results of this study showed that

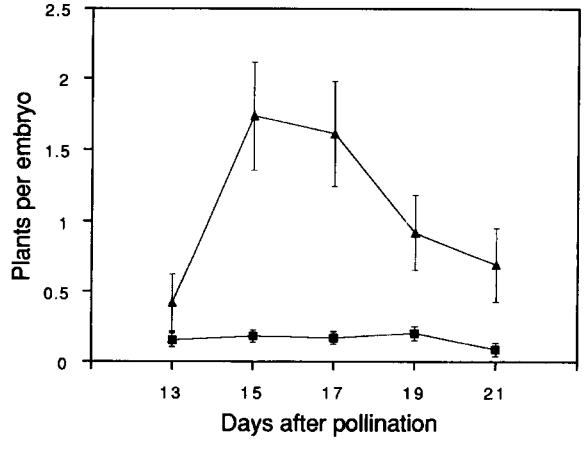


*Fig. 2.* Mean total plant production per plated embryo by days after pollination (± SE); ▲---, callus culture; ■---, embryo culture.

the hybrid embryos, depending on parental genotypes, varied in size and developmental stage and subsequently in their ability to develop into plants. In the field, the optimal date of rescue also depends on climatic conditions. This can be seen from the results of plant recovery from the two locations. The time range in both experiments for successful embryo rescue was over five days. The results in Experiment 1 suggest that rescue date earlier than 13 days after pollination could have been used successfully. However, in Experiment 2 the time range for successful embryo rescue was between 15 and 19 days. It would seem practical to rescue embryos nearer the later time limit when the embryos are larger. The observations on the continuous increase of the embryo size do not support the findings of Keyworth & Larter (1979), who reported that the tetraploid wheat-rye embryo volume ceased to increase 10–14 days after pollination. The observation that embryos of large size and good condition started to loose their ability to germinate or form callus after 17 days, indicates damage in the embryo before visible signs of abortion. A suitable rescue date would have been 15–17 days after pollination under both experimental conditions for all crosses, and for both culture methods. The range of optimal rescue time was narrower for callus culture than for embryo culture.

Merkle et al. (1988) reported that success in embryo and callus culture methods depended on the parental genotypes of the crosses. This study showed significant differences between the crosses but no medium × cross interaction in embryo development.

Callus culture showed potential for production of high numbers of hybrid plants from a single embryo. In addition, a larger number of embryos developed further after rescue on MS medium (by forming embryogenic callus) than on TL medium in both experiments. Improved plant regeneration methods, or repeated attempts to regenerate plants from embryogenic callus, would probably increase plant regeneration frequency and the total number of plants. Shao & Taira (1990) reported the potential of callus culture for producing hybrid plants from abnormal embryos. Their report does not, however, indicate the frequency of callus induction. In this study, callus culture showed potential to enhance development of abnormal embryos (in category 2) by promoting embryogenesis from larger numbers of embryos than germinated on embryo culture medium.

It is possible to maintain embryogenic callus through subcultures, thus allowing repeated opportunity for plant regeneration. In Experiment 1, regeneration ability was tested using only a single section of embryogenic callus, which may be the reason for the seemingly low regeneration ability in comparison with Experiment 2. In Experiment 2 all embryogenic callus was subcultured and the results demonstrate better the potential of callus culture for plant production. In both experiments callus was cultured for five weeks only. Subsequent subculturing might have increased the yield of hybrid plants. With five weeks of callus culture, the time necessary to produce amphihaploid plants was about six weeks longer than through embryo culture.

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