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# Effect of Genotype, Explant Age and Medium Composition on Callus Production and Plant Regeneration from Immature Embryos of Sorghum

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ABSTRACT. Regenerable callus was obtained from immature embryos of high-tannin sorghum (*Sorghum bicolor* L. Moench) genotypes. Immature embryos collected 1 to 2 weeks after pollination produced high frequency regenerable callus when cultured on basal medium containing Murashige and Skoog (MS) inorganic salts, modified Gamborg (B5) vitamins and supplemented with 2,4-D (2 mg/L). Coconut water (Cw, 10%) and/or zeatin (2.2 mg/L) promoted callus production in certain genotypes. Frequent subculturing on to the same medium under dark conditions reduced pigment formation. The genotypes differed in their ability to form callus as well as in subsequent regeneration. Shoots were obtained from nodular callus subcultured on the same basal medium supplemented with IAA (1.0 mg/L) and zeatin (2.2 mg/L). Casein hydrolysate, CH (1.0 g/L) enhanced shoot formation. The highest number of regenerated plants (70, 41, 18 and 17) was obtained in lines IS8768, G522A, BR64 and RTX430, respectively. These lines are rated high in tannin.

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**Keywords:** Immature embryos, High tannin sorghum, Tissue Culture.

**Abbreviations:**
2,4-D = 2,4-dichlorophenoxyacetic acid;
IAA = indoleacetic acid: CH = casein hydrolysate,
CW = coconut water.

Grain sorghum (*Sorghum bicolor* L. Moench), has recently been the subject of considerable research to improve its nutritional quality. Certain cultivars of sorghum which have a pigmented testa in the seed coat contain relatively high levels of condensed tannins which adversely affect the nutritional value of the grain (Butler *et al.* 1986). High tannin sorghums, however, have good agronomic characters such as relative resistance to bird depredation and preharvest germination (Butler 1989).

We are currently using tissue culture techniques to select clones of cells with unique characteristics of flavonoid metabolism which may be regenerated to obtain cultivars with improved nutritional or agronomic properties. We report here an effective protocol for callus formation and plant regeneration from genotypes of high tannin sorghum. Regeneration of sorghum *in vitro* has been attempted with variable success from various explants including mature sexual embryos and nodal tissue (Thomas *et al.* 1977 Cai *et al.* 1987), seedling segments (Mastellar and Holden 1970, Mascarenhas *et al.* 1975), immature embryos (Thomas *et al.* 1977, Gamborg *et al.* 1977, Dunstan *et al.* 1978, 1979), seedling leaves and segments of immature inflorescence (Wernicke and Brettell 1980, Brettell *et al.* 1980) and shoot-tips (Bhaskaran *et al.* 1988).

## Materials and Methods

Explants were obtained from inflorescences of sorghum inbred lines grown in the greenhouse (Line BR64) or at the Purdue University Agronomy Farm, West Lafayette, Indiana, season 1985/86, under the supervision of Dr. John Axtell.

Immature seeds were removed from the glumes of inflorescences at indicated times after flowering, sterilized in 70% ethanol for 5 to 7 minutes and then washed 3 times in sterile double distilled water. Immature embryos were excised from sterilized seeds in a laminar flow transfer hood and cultured on agar medium.

The embryo induction medium (pH 5.7) contained Murashige and Skoog (1962) inorganic salts, modified B5 vitamins (Gamborg *et al.* 1977), 20 g/L sucrose and 0.8% agar. Where indicated, the medium was supplemented (in mg/L) with 2,4-D (2.0 or 4.0), IAA (1.0), zeatin (2.2), casein hydrolysate (1000), and/or coconut water (CW) (the liquid endosperm of the coconut), 10% v/v. Cultures were maintained either in the dark or in a walk-in growth chamber with a 16h light/8h dark cycle under cool white fluorescent lamps at an intensity of 50-80 $\mu$ mol $m^{-2}$ $s^{-1}$ and at 26°C.

## Results

*Immature Embryo Explants*

Immature embryos from a high tannin hybrid sorghum, DeKalb BR64, grown in the greenhouse were harvested at 1, 2, 3 and 4 weeks after pollination. The average length of the immature embryos was 0.5, 1.0, 2.5 and 3.0 mm, respectively.

Embryos obtained 2, 3, and 4 weeks after pollination and cultured on medium containing MS salts, 2 or 3% sucrose, modified B5 vitamins, and ± casein hydrolysate, germinated without callus production after 3-5 days in culture. Embryos obtained 1 week after pollination neither germinated nor formed callus on this medium. Embryos started to form callus after 2-3 weeks on medium supplemented with 2,4-D alone or in combination with zeatin or coconut water. Callus was formed either directly on the scutellum, *i.e.* from embryonic tissue, or at the base of a short extruded plumule. Callus that formed directly on the scutellum was compact, nodular, yellowish white and resembled "popcorn". This type of callus was produced at a higher frequency from immature embryos 1-2 weeks after pollination and can be maintained by subculture on the same induction medium in the dark, without pigment production. The second type of callus that formed on the plumule was translucent, friable and produced roots only on regeneration medium.

Table 1 indicates that callus production was highest from embryos excised 2 weeks after pollination and cultured on media with 2,4-D or 2,4-D + CW (10%) in the dark. Immature embryos excised 2 or 3 weeks after pollination and cultured in the light produced pigments in all treatments and formed callus of the second type. Light also reduced the amount of callus produced.

Callus that formed directly on the scutellum of immature embryos was tested for regeneration on 4 potential regeneration media in the light (Table 2). Multiple leafy shoots were formed after 2 weeks on medium containing IAA, and zeatin. Casein hydrolysate enhanced shoot formation but only in the presence of IAA and zeatin. 2,4-D (2 mg/L) had no effect on shoot formation. Shoots recovered from the regeneration medium continued to grow and upon transfer to medium without hormones, they produced roots and elongated extensively. They were then potted in a mixture of vermiculite, sand, and peatmoss and transferred to the greenhouse.

On the basis of these results, the experiment was repeated using field grown sorghum. Immature embryos were collected 2 weeks after pollination from 14 sorghum lines differing in their tannin or phenolic content according to Price *et al.* 1978. Cultures were incubated in a growth chamber under dark conditions.

Callus production occurred within 2-4 weeks depending on genotype. Data taken after 4 weeks (Table 3) indicates that callus production was influenced by genotype and medium composition. The same medium (2,4-D supplemented with zeatin and coconut water) gave the highest (100% with G522A) and lowest (0% with 3 lines) % of explants forming callus. Although 2,4-D alone (2 mg/L) induced callus formation in all lines tested, the addition of CW (10% v/v) and zeatin (2.2 mg/L) promoted callus production only in highly pigmented lines, *e.g.* BR64 and G522A while CW alone tended to reduce callus production in general. Relatively high concentrations of 2,4-D (4 mg/L) in the presence of CW reduced callus production in most lines. In previous experiments low 2,4-D concentration (1 or <1 mg/L) reduced callus growth and enhanced root formation.

Upon transfer of callus to regeneration medium (3% sucrose, B5 vit., casein hydrolysate, IAA and zeatin) the lines differed markedly in their ability to regenerate plants (Table 4). The frequency of callus clones that regenerated plants was highest in lines IS8768 (22%) RTX430 (22%) and G522A (16%); the number of regenerated plants in these lines after 1 to 2 months on regeneration medium was 70, 17 and 41 respectively. The other lines generally regenerated fewer (0-14%) plants.

**Table 1.** Callus production from immature embryos of BR64 cultured for four weeks (25 explants per treatment)

| Culture Condition | Medium* | Callus Production (%) | | | | Mean |
|---|---|---|---|---|---|---|
| | | Weeks after Pollination | | | | |
| | | 1 | 2 | 3 | 4 | |
| Dark | BM | 0 | 0 | 0 | 0 | 0 |
| | 2,4-D | 15 | 32 | 25 | 30 | 25.5 |
| | 2,4-D + Z | 20 | 20 | 10 | 10 | 15 |
| | 2,4-D + CW | 25 | 36 | 25 | 15 | 25 |
| Mean | | 15 | 22 | 15 | 13.5 | |
| Light | BM | 0 | 0 | 0 | – | 0 |
| | 2,4-D | 10 | 48 | 8 | – | 22 |
| | 2,4-D + Z | 20 | 12 | 8 | – | 13 |
| | 2,4-D + CW | 20 | 4 | 8 | – | 14 |
| Mean | | 12.5 | 16 | 6 | – | |

* BM = basal medium (MS inorganic salts + B5 vit. + sucrose)
2,4-D (2 mg/L); z = zeatin (2.2 mg/L); CW (10% v/v).

## Discussion

Callus initiated from immature embryos was regenerable and resembled that described by Gamborg *et al.* (1977) in sorghum and Hanzel *et al.* (1985) in certain genotypes of barley. The age of the embryo influenced the production of regenerable callus in BR64. Embryos that were 0.5-1.0 mm (1-2 weeks after pollination) produced the desired type of callus. This was also noticed in maize (Green and Phillips 1975), barley (Dale and Deambrogio 1979, Hanzel *et al.* 1985) and wheat (Sears and Deckard 1982). Although, callus formation and subsequent plant regeneration have occured on media of composition similar to that of Gamborg *et al.* (1977), strong genotypic differences among 14 sorghum lines were noticed. For example, the mean frequency of callus formation on 4 different medium combinations varied between 66 and 7% depending on the genotype (Table 3). In addition, each line responded differently to each of the 4 medium combinations indicating that for each genotype a specific medium is favored. For instance, line G522A had 100% callus formation on medium containing 2,4-D (2 mg/L), CW and zeatin, and only 67, 43 and 37% on I, II and III medium

**Table 2.** Effect of medium composition on shoot production from callus of immature embryos of BR64

| Composition of embryo induction medium[z] | Hormones & other additives[+] | | | | Shoot formation (0=none, øøø=extensive) |
|---|---|---|---|---|---|
| | 2,4-D (2 mg/L) | IAA (1 mg/L) | Z (2.2 mg/L) | CW (10% v/v) | |
| 2% sucrose, B5 | – | – | – | – | 0 |
| 2% sucrose, B5, CH | – | – | – | – | 0 |
| 3% sucrose, B5 | – | – | – | – | 0 |
| 3% sucrose, B5, CH | – | – | – | – | 0 |
| 2% sucrose, B5 | + | – | – | – | 0 |
| 2% sucrose, B5 | + | – | + | – | 0 |
| 2% sucrose, B5 | + | – | + | + | 0 |
| 2% sucrose, B5 | – | + | + | – | ø |
| 2% sucrose, B5, CH | – | + | + | – | øø |
| 3% sucrose, B5 | – | + | + | – | ø |
| 3% sucrose, B5, CH | – | + | + | – | ööö |

[z]The basal medium contained Murashige and Skoog inorganic salts.

B5 = modified B5 vitamin (B5 vit. according to Gamborg (1975) enriched with mg/L: L. asparagine, 200; glycine, .7; calcium pantothenate, 0.25; and niacinamide, 1.3).

CH = casein hydrolysate (1 g/L).

[+]indicates hormone or additive included, – indicates hormone or additive not included.

combinations respectively. Several other lines failed to produce callus on medium containing 2,4-D, CW and zeatin (Shawaya, SC0167-14E, IS8260) though they formed callus at low frequency when zeatin was not included.

Genotypic differences with respect to callus initiation were reported in barley by Dale and Deambrogio (1979) and by Hanzel *et al.* (1985). Hanzel *et al.* (1985) also noticed significant genotype x medium interactions for callus initiation. While such interactions in the present study cannot be excluded, attention should be given to the concentration of 2,4-D and the type and concentration of vitamins and amino acids in the callus induction medium. In preliminary experiments 2 mg/L, 2,4-D was found to be optimum for callus growth and inhibition of root formation. This concentration was also found to be optimum for callus growth in wheat (Ozias-Akins and Vasil 1982). Supplementing the media with modified B5 vitamins (Gamborg *et al.* 1977) was found superior to MS vitamins for the induction of regenerable callus (data not shown). The addition of coconut water and zeatin was

**Table 3.** Callus production from immature embryos of 14 inbred lines of sorghum after 4 weeks in culture

| Line | % callus production treatments* | | | | Total No. of explants | Mean % of explants that formed callus |
|---|---|---|---|---|---|---|
| | I | II | III | IV | | |
| RS610 | 27 | 20 | 0 | 35 | 88 | 20.5 |
| 8768 | 26 | 26 | 13 | 22 | 109 | 21.8 |
| SAVIII | 16 | 34 | 26 | 18 | 177 | 23.5 |
| BR64 | 13 | 12 | 13 | 23 | 134 | 15.3 |
| G522A | 6 | 43 | 37 | 100 | 82 | 61 |
| 4225 | 87 | 40 | 50 | 70 | 59 | 61.8 |
| SHAWAYA | 8 | 22 | 11 | 0 | 8 | 10.3 |
| P954035 | – | 35 | 16 | 41 | 56 | 30.6 |
| SC0167-14E | 16 | 0 | 10 | 0 | 60 | 6.5 |
| ORO | 66 | 50 | 47 | 50 | 64 | 53.3 |
| 2319 | 63 | 63 | 55 | 37 | | 54.5 |
| 0469 | 50 | 31 | 33 | 11 | 65 | 31.3 |
| IS8260 | 14 | 31 | 23 | 0 | 46 | 1.5 |
| RTX430 | 83 | 66 | 66 | 50 | 24 | 66.3 |
| Mean | 38.3 | 33.8 | 28.5 | 32.6 | | |

*The treatments contained Murashige and Skoog inorganic salts and B5 vitamins as basal medium and the following:

I = 2.0 mg/L 2,4-D
II = 2.0 mg/L 2,4-D + 10% CW
III = 4.0 mg/L 2,4-D + 10% CW
IV = 2.0 mg/L 2,4-D + 10% CW + 2.2 mg/L Zeatin

not required for callus induction but promoted the process in certain genotypes (Table 3), and may influence further differentiation.

Pigment formation, frequently encountered in sorghum cultures (Gamborg *et al.* 1977, Dunstan *et al.* 1979, Davis and Kidd 1980, was much minimized under our conditions. We were able to induce callus which eventually regenerated plants from immature embryos of sorghum lines that are considered highly pigmented. In fact, most of our regenerated plants were obtained from highly pigmented genotypes, *e.g.* IS8768, G522A and BR64.

As with callus formation, genotypic differences with respect to shoot formation were also encountered. With a given medium, there were differences among the 14 genotypes for their ability to regenerate plants (Table 4). These results are in agreement with the observation made by Hongtu Ma and Liang (1983) in sorghum and with the results of Green and Phillips (1975) in maize, Dale and Deambrogio (1979) and Hanzel *et al.* (1985) in barley, Sears and Deckard (1982) in wheat, and Rines and McCoy (1981) in oats.

However, unlike barley (Hanzel *et al.* 1985) or wheat (Ozias-Akins and Vasil 1982) shoot formation in sorghum did not occur upon lowering or omitting the 2,4-D from the medium. Shoot formation occured upon transfer to regeneration

**Table 4.** Plant regeneration from callus induced from immature embryos of 14 sorghum genotypes

| Line | Regenerating clones | | No. of regenerated plants[z] | Intensity |
|---|---|---|---|---|
| | No. | % | | |
| RS610 | 2 | 10 | 11 | 5.5 |
| 8768 | 6 | 22 | 70 | 11.6 |
| SAVIII | 2 | 4 | 5 | 2.5 |
| BR64 | 4 | 13 | 18 | 4.5 |
| G522A | 7 | 16 | 41 | 5.8 |
| 4225 | 2 | 6 | 5 | 2.5 |
| SHAWAYA | 0 | 0 | 0 | 0 |
| P954035 | 3 | 14 | 4 | 1.3 |
| SC0167-14E | 0 | 0 | 0 | 0 |
| ORO | 3 | 8 | 7 | 2.3 |
| 2319 | 3 | 7 | 14 | 4.6 |
| 0469 | 1 | 4 | 2 | 2.0 |
| IS8260 | 1 | 13 | 2 | 2.0 |
| RTX430 | 4 | 22 | 17 | 4.3 |

[z]After 1-2 months (2 subcultures) on regeneration medium.

medium containing IAA and zeatin and was enhanced in the presence of casein hydrolysate. Further evaluation of the medium components and their interactions with genotype should improve regeneration from callus of high tannin sorghum.

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# تأثير الطراز الوراثي وعمر الجزء المستزرع والبيئة الغذائية على تكوين كدب ونباتات من أجنة ذرة غير مكتملـــة النمـــو

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محصول الذرة من الحبوب التي تحتل مكـانة مـرموقـة في الاستهلاك والانتـاج العـالمي بعد القمـح والارز والذرة الشـامية والشعـير. معظم السـلالات المنتجـة تحتوي على مواد قابضة (condensed tannins) مما يقلل الفـائدة الغـذائية لهـا. لذلـك تجرى أبحاث متعددة بغرض تحسين الفائدة الغذائيـة مع المحـافظة عـلى الصفات الحقلية الجيدة.

في هذا البحث استخدمت تقنية زراعة الأنسجة النباتية لدراسة إمكانية انتاج كدب (Callus) ومن ثم نباتات من عدة سلالات من الذرة المحتوية على نسبة عالية من المواد القابضة. جمعت حبوب الذرة من محصول حقلي في اطوار نمو مختلفة بعـد التلقيح. بعد التعقيم في المعمل استخرجت الاجنة من الحبوب وزرعت عـلى بيئة غـذائية معقمـة تتكـون من وسـائط مـوراشيجي واسكـوج (MS) كبيئـة اسـاسيـة بالاضافة لعدة عوامل غذائية وهرمونية أخرى لمعرفة تأثيرها على افراز كدب منتـج للنباتات.

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كليـة الصيدلـة ـ جامعـة الملك سعود ـ ص.ب: ٢٤٥٧ ـ الـرياض ١١٤٥١ ـ المملكـة العربية السعودية.

وجد أنه لابد من اضافة هرمون 2,4 - D (بتركيز ٢ مج / لتر) للبيئة الاساسية لتكوين كدب من الأجنة في المرحلة الأولى. وان الأجنة التي أخذت بعد أسبوع أو أسبوعين بعد التلقيح هي الأكثر قدرة على إفراز كدب من النوع المرغوب على هذه البيئة الغذائية. وعند نقل الكدب لبيئة غذائية حديثة التكوين وجد أنه لابد من اضافة هرمون IAA و Zeatin لتكوين نباتات من الكدب كما لوحظ تبايناً واضحاً بين السلالات في مقدرتها على افراز كدب ومن ثم نباتات وان السلالات IS8768 ، G522A ، BR 64 ، RTX430 هي الأكثر إنتاجاً للنباتات مع أنها تحتوي على نسبة عالية من المواد القابضة.