In Vitro Cell. Dev. Biol.—Plant 39:161–164, March–April 2003
DOI: 10.1079/IVP2002385

1054-5476/03 $18.00+0.00

HIGH COPPER LEVELS IMPROVE CALLUS INDUCTION AND PLANT REGENERATION IN *SORGHUM BICOLOR* (L.) MOENCH

R. S. NIRWAN AND S. L. KOTHARI*

Department of Botany, University of Rajasthan, Jaipur-302 004, India

(Received 31 May 2002; accepted 16 October 2002; editor C. J. Chetsanga)

Summary

A highly efficient protocol for callus induction and plant regeneration in *Sorghum bicolor* was developed by varying the concentrations of copper (0.1, 0.3, 0.5, 0.7, 1, 1.5, 2, 5 μM) in Murashige and Skoog (MS) medium. The mature embryos of *Sorghum bicolor* were cultured on MS medium containing 2,4-dichlorophenoxyacetic acid (9 μM), kinetin (2.3 μM), and 3% (w/v) sucrose for embryogenic callus induction. Plant regeneration from this callus occurred on MS medium containing kinetin (9.2 μM) and indole-3-acetic acid (2.85 μM). A much greater response was noted on these media with higher levels of copper. Frequency of plant regeneration and number of regenerants dramatically increased with an optimal amount of copper (2 μM) in the MS medium. Rooting of the regenerated shoots readily occurred on half-strength MS medium supplemented with α-naphthaleneacetic acid (10.7 μM) and 3% (w/v) sucrose. Well-developed plantlets were transferred to the field where 100% survival and normal seed setting was noted.

Key words: mature embryos; copper sulfate; plant growth regulator.

Introduction

Sorghum bicolor (L.) Moench ranks fifth most planted crop amongst cereals and millets in the world. It is the primary staple food crop of arid and semi-arid zones of the world. Species of this genus are sources of grain, fiber, fuel, and secondary products (Lusardi and Lupotto, 1990). Development of efficient regeneration protocols is a prerequisite for the application of transformation technology. *Sorghum* has been categorized as one of the most difficult plant species to manipulate in tissue culture and transformation experiments (Zhu et al., 1998). In *Sorghum bicolor* different explants have been used for *in vitro* studies, including immature embryos (Gamborg et al., 1977; Ma et al., 1987; Sharma et al., 1989), mature embryos and fully-developed inflorescences (Thomas et al., 1977), immature inflorescences (Brettell et al., 1980), shoot apices (Nahdi and de Wet, 1995; Zhong et al., 1998), and leaves (Wernicke and Brettell, 1980, 1982).

Media manipulation and explant choice (Smith and Bhaskaran, 1986) are still the best variables under experimental control for successful plant regeneration from the different cultivars. Inorganic macronutrient and micronutrient levels used in most plant tissue culture media are based on levels established in a medium (MS) developed by Murashige and Skoog (1962) for tobacco tissue culture. Recent studies on wheat and barley by Purnhauser (1991) and Dahleen (1995) have shown that an increased level of copper dramatically improves regeneration. The objective of this study was to evaluate the effect of different copper levels in MS medium on callus induction and plant regeneration in sorghum. Our protocol for sorghum shows several-fold higher efficiency both in callus induction and plantlet regeneration. This protocol will help in increasing the probability of obtaining transgenic sorghum plants at higher efficiency.

Materials and Methods

Seeds of *Sorghum bicolor* (L.) Moench var. FSH-4 were procured from Mahyco, India. These seeds were thoroughly washed and surface-sterilized with 70% ethanol for 1 min and subsequently 3 min in a 1% aqueous solution of $HgCl_2$. Surface-sterilized seeds were rinsed with double-distilled water three times. Sterilized seeds were soaked for 40 h in double-distilled water at a temperature of 26°C before excision of mature embryos. Mature embryos were aseptically dissected from seeds and placed with the scutellum upward on solid medium containing MS mineral salts and different concentrations of copper sulfate. The levels of copper were varied up to 50 times that in MS medium in order to observe the effect of copper on callus induction and regeneration. The various levels of copper sulfate used were 0.1, 0.3, 0.5, 0.7, 1, 2, 5 μM. Plant growth regulators – 2,4-dichlorophenoxyacetic acid (2,4-D; 9.0 μM), kinetin (2.3 μM), and sucrose (3% w/v) – were added. The pH was adjusted to 5.8 and the medium was solidified with 0.8% agar (Qualigens, India). The medium was autoclaved at 121°C and 1.06 kg cm^{-2} pressure for 15 min. All the cultures were incubated in a growth chamber at a temperature of 26 ± 1°C and 16 h photoperiod (25 $\mu mol\ m^{-2}\ s^{-1}$). Visual observations were made for up to 4 or 5 wk. Fifty explants were used for each treatment.

The primary embryogenic callus derived from mature embryos was transferred to regeneration medium RM_1 [MS medium supplemented with kinetin (9.2 μM), indole-3-acetic acid (IAA; 2.85 μM), and copper sulfate (0.1 μM)] and on modified regeneration medium RM_2 [MS medium supplemented with kinetin (9.2 μM) and IAA (2.85 μM)] and higher levels of copper sulfate. After 4 wk of inoculation, observations were recorded. Healthy green shoots were transferred to rooting medium [half-strength MS medium supplemented with α-naphthaleneacetic acid (NAA; 10.7 μM)] and sucrose 3% (w/v). Plantlets with well-developed root and shoot systems were transferred to earthen pots containing soil and compost in the ratio of 1:1. Acclimatization was not required before transferring the plantlets to the field.

*Author to whom correspondence should be addressed: Email SLKothari@Lycos.com

Results and Discussion

Callus initiation frequency from mature embryos of sorghum ranged from 85 to 100% on MS medium supplemented with 2,4-D (9.0 μM) plus kinetin (2.3 μM) (Fig. 1a_I) and various levels of copper sulfate (Table 1). Callus was compact, nodular, embryogenic with a smooth shiny surface along with a soft, friable, loosely-packed and non-embryogenic type of callus. Callus induction and plant regeneration response was highly dependent on the concentration of copper sulfate in the MS medium. Callusing responses on the medium devoid of copper sulfate was very poor. On increasing the concentration of copper sulfate, the amount of callus dramatically increased and was optimized on 2 μM copper sulfate (Fig. 1a_{II}).

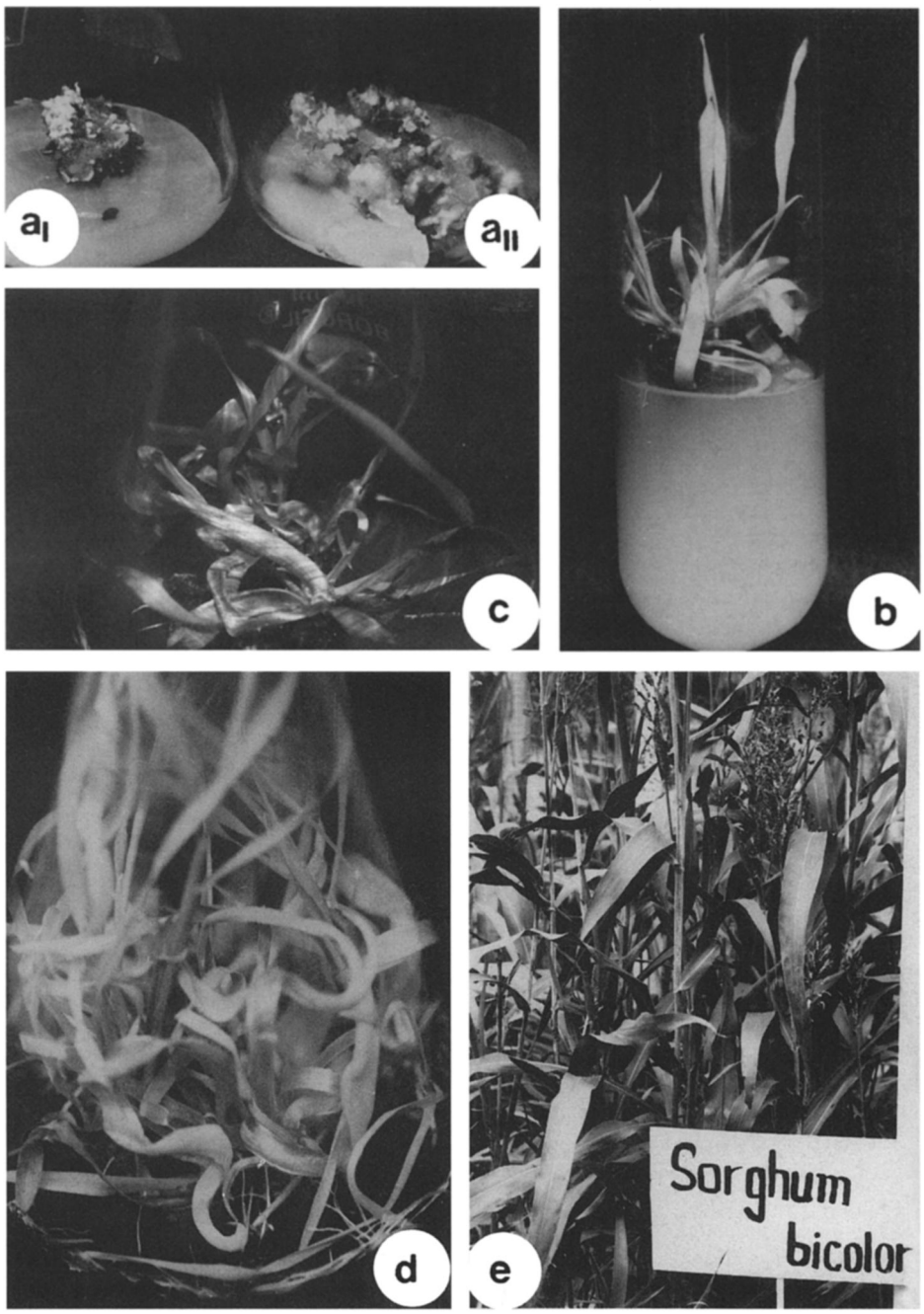

Fig. 1. Effect of copper on callus induction and plant regeneration in *Sorghum bicolor* (L.) Moench. a_I, Callus induction on MS + 2,4-D (9 μM) + kinetin (2.3 μM); a_{II}, callus induction on MS + 2,4-D (9 μM) + kinetin (2.3 μM) with 2 μM $CuSO_4$; *b*, regeneration from embryogenic callus on plain MS medium; *c*, regeneration from embryogenic callus on MS + kinetin (9.2 μM) + IAA (2.85 μM); *d*, regeneration from embryogenic callus on MS + kinetin (9.2 μM) + IAA (2.85 μM) with 2 μM $CuSO_4$; *e*, field-transferred plants of *Sorghum bicolor*.

TABLE 1

MORPHOGENETIC RESPONSE OF MATURE EMBRYOS OF *SORGHUM BICOLOR* CULTURED ON MS MEDIUM SUPPLEMENTED WITH KINETIN (2.3 μ*M*) + 2,4-D (9 μ*M*) AND DIFFERENT LEVELS OF COPPER

Copper levels in medium (μ*M*)	Callus induction frequency (%)	Fresh weight (g) (mean ± SD)[z]
0	85	0.49 ± 0.09 a
0.1	90	0.77 ± 0.10 bf
0.3	94	0.85 ± 0.03 b
0.5	90	0.75 ± 0.03 bf
0.7	95	1.16 ± 0.60 c
1.0	100	1.15 ± 0.25 c
1.5	100	2.14 ± 0.45 d
2.0	100	3.46 ± 0.50 e
5.0	87	0.70 ± 0.02 f

[z] Different *letters* following each value within a *column* indicate significant difference by Fisher's protected LSD ($P < 0.05$).

The percentage of embryos capable of producing regenerable cultures is an important trait affecting the efficiency of sorghum transformation. Regeneration response of embryogenic callus on plain MS medium was less prominent (Fig. 1*b*) compared to the response on RM_1 medium (Fig. 1*c*). The number of plantlets regenerated per culture increased with the increasing levels of copper sulfate in the regeneration medium (Table 2). Visual comparison of results revealed that higher copper levels as in RM_2 dramatically increased the number of regenerants. Embryogenic callus derived from MS medium supplemented with 2 μ*M* copper sulfate was transferred to regeneration medium RM_2, where it gave two and half times more regenerants (Fig. 1*d*). The regenerated plants developed normal seeds in the field (Fig. 1*e*).

Evaluation studies of regenerated plants per embryo have been conducted in barley by Baillie et al. (1993) where they obtained 0.34 plants. Luhrs and Lorz (1987) found a range of 0.4–2.7 plants per embryo in their study of 36 genotypes of barley. After modifying the copper levels in the medium, Luhrs and Lorz (1987) were able to obtain an average of 22.3 plants per embryo. In *Hordeum vulgare*, Dahleen (1995) obtained 41 plants per embryo culture and in this study of sorghum 61 plants per embryo culture have been obtained.

The exact basis for this is unknown but it has been suggested that 1–25 μ*M* copper sulfate in a hydroponic nutrient solution inhibits the formation of the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid and, thus, promotes regeneration (Lidon et al., 1995). Purnhauser and Gyulai (1993) suggested that it is not through the ethylene-inhibiting action that copper ions promote regeneration, since copper ions are components or activators of many enzymes involved in electron transport, protein and carbohydrate biosynthesis, and polyphenol metabolism. It is speculated that some copper enzymes might play an important role in regeneration.

The results of the present study showed that copper levels in MS medium were not optimal for callus induction and subsequent plant regeneration in *Sorghum bicolor*. Optimization of the copper level resulted in efficient callus induction and higher-frequency plant regeneration.

TABLE 2

REGENERATION RESPONSE OF EMBRYOGENIC CALLUS DERIVED FROM MATURE EMBRYOS OF *SORGHUM BICOLOR*

Copper levels in callus induction medium (μ*M*)	Mean number of regenerants per callus culture	
	RM_1 (mean ± SD)[z]	RM_2 (mean ± SD)[z]
0.1	0.0 ± 1.6 b	8.0 ± 1.6 a
0.3	9.4 ± 2.7 cdf	10.4 ± 2.6 b
0.5	9.0 ± 2.4 bcf	13.0 ± 2.9 c
0.7	10.2 ± 3.7 def	13.2 ± 3.1 c
1.0	10.6 ± 2.5 e	15.8 ± 1.3 d
1.5	9.4 ± 4.1 f	15.6 ± 3.4 d
2.0	10.6 ± 1.6 e	17.6 ± 3.6 e

RM_1 = MS medium + kinetin (9.2 μ*M*) + IAA (2.85 μ*M*) + copper (0.1 μ*M*).

RM_2 = MS medium + kinetin (9.2 μ*M*) + IAA (2.85 μ*M*) + copper levels as in callus induction medium.

[z] Different *letters* following each value within a *column* indicate significant difference by Fisher's protected LSD ($P < 0.05$).

Acknowledgments

We thank ICAR, New Delhi, for sponsoring a scheme on small millets and for a fellowship to R. S. Nirwan.

References

Baillie, A. M. R.; Rossnagel, B. G.; Kartha, K. K. Evaluation of 10 Canadian barley (*Hordeum vulgare* L.) cultivars for tissue culture response. Can. J. Plant Sci. 73:171–174; 1993.

Brettell, R.; Wernicke, W.; Thomas, E. Embryogenesis from cultured immature inflorescences of *Sorghum bicolor*. Protoplasma 104:141–148; 1980.

Dahleen, L. S. Improved plant regeneration from barley callus cultures by increased copper levels. Plant Cell Tiss. Organ Cult. 43:267–269; 1995.

Gamborg, O. L.; Shyluk, J. P.; Brar, D. S.; Constabel, F. Morphogenesis and plant regeneration from callus of immature embryos of sorghum. Plant Sci. Lett. 10:67–74; 1977.

Lidon, F. C. M.; Da Graca Barreiro, M.; Santos Henriquest, F. Interactions between biomass production and ethylene biosynthesis in copper treated rice. J. Plant Nut. 18:1301–1314; 1995.

Luhrs, R.; Lorz, H. Plant regeneration *in vitro* from embryogenic cultures of spring and winter-type barley (*Hordeum vulgare* L.) varieties. Theor. Appl. Genet. 75:16–25; 1987.

Lusardi, M. C.; Lupotto, E. Somatic embryogenesis and plant regeneration in sorghum species. Maydica 35:59–66; 1990.

Ma, H.; Gu, M.; Liang, G. H. Plant regeneration from cultured immature embryos of *Sorghum bicolor* (L.) Moench. Theor. Appl. Genet. 73:389–394; 1987.

Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473–497; 1962.

Nahdi, S.; de Wet, J. M. J. *In vitro* regeneration of *Sorghum bicolor* lines from shoot apices. Int. Sorghum Millets Newsl. 36:88–90; 1995.

Purnhauser, L. Stimulation of shoot and root regeneration in wheat (*Triticum aestivum*) callus cultures by copper. Cereal Res. Commun. 19:419–423; 1991.

Purnhauser, L.; Gyulai, G. Effect of copper on shoot and root regeneration in wheat, triticale, rape and tobacco tissue cultures. Plant Cell Tiss. Organ Cult. 35:131–139; 1993.

Sharma, V.; Kothari, S. L.; Chandra, N. *In vitro* regeneration, field transfer of plantlets and growth to maturity of plants of *Sorghum bicolor* (L.). Moench. Curr. Sci. 58:586–588; 1989.

Smith, R. H.; Bhaskaran, S. Sorghum (*Sorghum bicolor* (L.) Moench).

In: Bajaj, Y. P. S., ed. Biotechnology in agriculture and forestry crops I. New York: Springer-Verlag; 1986.

Thomas, E.; King, P. J.; Potrykus, I. Shoot and embryo like structure formation from cultured tissues of *Sorghum bicolor*. Naturwissenschaften 64:587; 1977.

Wernicke, W.; Brettell, R. I. S. Somatic embryogenesis from *Sorghum bicolor* leaves. Nature 287:138–139; 1980.

Wernicke, W.; Brettell, R. I. S. Morphogenesis from cultured leaf tissue of *Sorghum bicolor*. Culture initiation. Auxins. Protoplasma 111:19–27; 1982.

Zhong, H.; Wang, W.; Sticklen, M. *In vitro* morphogenesis of *Sorghum bicolor* (L.) Moench. Efficient plant regeneration from shoot apices. J. Plant Physiol. 153:719–726; 1998.

Zhu, H.; Muthukrishna, S.; Krishnaveni, S.; Wild, G.; Jeoung, J. M.; Liang, G. H. Biolistic transformation of sorghum using a rice chitinase gene. J. Genet. Breed. 52:243–252; 1998.