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Improved plant regeneration from maize callus cultures using 6-benzylaminopurine

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ABSTRACT

A new protocol for regenerating plants from cultured type I callus of the maize (Zea mays L.) inbred Pa91 includes growing the callus on medium containing 3.5 mg/l (15.5 μM) of the cytokinin 6-benzylaminopurine (6BA) for 3 to 6 d and then moving the callus to medium containing no growth regulators (H medium) for an additional 15 to 21 d, where the plants actually develop. The number of plants regenerated from the 6BA treated callus was 113% to 148% greater than the number of plants produced from callus placed directly on H medium. This increased plant regeneration induced by 6BA seemed to maximize the number of plants regenerated from a gram of callus and was slightly affected by callus age or prior treatment of callus with $AgNO_3$. Exposure to 6BA for 9 d greatly reduced shoot and root development, and longer exposures totally prevented root formation. This inhibition of root formation could be reversed only slightly by naphthaleneacetic acid. The data indicate that high concentrations of 6BA are effective for increasing plant regeneration from maize callus cultures when short exposure times are used. This procedure has also been effective for regenerating many plants from the inbreds H99 and Mo17.

ABBREVIATIONS: 6-Benzylaminopurine, 6BA; indole-3-acetic acid, IAA; Naphthaleneacetic Acid, NAA; 2,4-dichlorophenoxyacetic acid, 2,4-D; 3,6-dichloro-o-anisic acid, dicamba; Gibberellic acid, GA_3; gram fresh weight, gfw.

INTRODUCTION

Since the introduction of immature embryos as donor tissues for establishing callus cultures of maize (Green and Phillips, 1975), plant regeneration from such cultures has become a common practice and many methods for regenerating plants have been published. Most methods (an exception is Fahey et al., 1986) use either a Murashige and Skoog (1962) based medium or an N_6 based medium (Chu et al., 1975), and plant regeneration is typically induced by removing the auxin source (Green et al., 1983; Armstrong and Green, 1985; Duncan et al., 1985). Reported variations of this procedure include the addition, deletion or alteration in concentration of chemicals in the medium and each variation seems to be developed specifically for the particular genotype used in a study. For instance, L-proline has been added and deleted from media to show that it has a positive effect on culture and plant production (Armstrong and Green, 1985; Duncan and Widholm, 1987a). Sugar concentrations in regeneration medium have been elevated and lowered with variable effects on plant regeneration (Green et al., 1983; Lu et al., 1983; Malmberg et al., 1984). Also the addition of $AgNO_3$ to the regeneration medium has been reported to increase plant regeneration (Duncan and Widholm, 1987b; Songstad et al., 1988).

The most commonly reported difference in regeneration protocol is the type and concentration of growth regulator(s) in the regeneration medium. Indole-3-acetic acid (IAA) or the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) have been added to regeneration media in concentrations from 0.1 to 1.0 mg/l and in combination with abscisic acid (Green et al., 1983), a cytokinin (Chang, 1983; Vasil et al., 1983), or a cytokinin plus gibberellic acid (Radojevic, 1985) with variable effects on plant regeneration. Gibberellic acid (GA_3) has been added to regeneration medium in concentrations from 1.0 to 2.0 mg/l, alone (Novak et al., 1983; Lu et al., 1983) or in combination with a cytokinin and IAA (Radojevic, 1985), with little or no increase in plant regeneration.

Maize regeneration media have been amended most often with cytokinins, in concentrations from 0.1 to 10 mg/l, as kinetin (Harms et al., 1976; Vasil et al., 1983; Kamo et al., 1985; Lowe et al., 1985; Radojevic, 1985), zeatin (Chang, 1983; Radojevic, 1985), 6-benzylaminopurine (Vasil et al., 1983), 6-(γ,γ-dimethylallylamino)purine (Torne et al., 1980; Torne et al., 1984; Santos et al., 1984) or 10% coconut milk (Fahey et al., 1986) which contains zeatin derivatives. These reports show that the effect of cytokinins on plant regeneration in maize vary from having no apparent effect (Harms et al., 1976; Vasil and Vasil, 1984; Kamo et al., 1985) to being stimulatory to shoot production (Lowe et al., 1985; Radojevic, 1985).

We have often observed embryoids on callus pieces on regeneration medium that do not develop into whole plants. These observations suggest that there is a greater capacity for plant production from maize cultures than has been realized using the

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present protocol (Duncan et al., 1985). The above mentioned literature suggests that, of the growth regulators tested, a cytokinin added to regeneration medium might be useful for improving plant regeneration. However, these reports vary greatly in terms of the concentrations used and the combining of a cytokinin with other growth regulators. These reports are also contradictory in terms of the benefits resulting from the use of a cytokinin. Because of these contradictions, there is no clear and usable protocol for the application of a cytokinin to plant regeneration. In fact, many of these reports just mention, as standard procedure, that a cytokinin was used. The results presented here are directed towards clarifying the use of a cytokinin in the regeneration of plants from maize tissue cultures and show that, with proper application, at least 6-benzylaminopurine can greatly increase plant regeneration from maize tissue cultures.

MATERIALS AND METHODS

Culture Development. Type I maize (Zea mays L.) calli capable of plant regeneration were developed from immature embryos of the self-pollinated inbreds H99, Mo17 and Pa91 as described by Duncan et al. (1985). Callus was maintained in the dark at 28°C on D medium (Duncan et al., 1985) and only callus pieces regenerable in appearance and of approximately 0.01 gfw were subcultured to fresh D medium at 21-d intervals.

Plant regeneration. Plants were regenerated by placing 20 pieces of randomly chosen, regenerable callus, approximately 0.2 gfw total, on one fourth of a 100 X 25 mm Petri plate containing 28 ml of H medium (Duncan et al., 1985) and then wrapping the plates in two layers of parafilm. Modification of this procedure consisted of using callus of different ages or adding various concentrations of 6-benzylaminopurine (6BA), naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) or 3,6-dichloro-o-anisic acid (dicamba) to the H medium. Other protocol alterations consisted of growing callus on D medium containing 200 μM $AgNO_3$ for one subculture prior to transferring the callus to H medium (Duncan and Widholm, 1987b). Application times of these media modifications are described in the Results and Discussion. Plants were grown to maturity as previously described (Duncan et al., 1985). Experiments with Pa91 were repeated four times over a nine month period. Results from one representative experiment are shown in Table 1.

RESULTS AND DISCUSSION

To determine the effect of 6BA on plant regeneration from cultures of Pa91, callus was exposed for 21 d to 6BA concentrations from 0.35 mg/l to 4.0 mg/l (1.55 to 17.8 μM) in the regeneration medium (data not included). In this preliminary experiment, no noticeable difference in plant regeneration was detected between the control treatment lacking 6BA and concentrations of 6BA less than 3.5 mg/l (15.5 μM). Plant regeneration was substantially increased at 6BA concentrations of 3.5 and 4.0 mg/l, and no visible differences were noticed between these two treatments. No roots were produced on the regenerated plantlets which were exposed for 21 d to the highest levels (3.5 and 4.0 mg/l) of 6BA (exemplified by Mo17 in Fig. 1). This

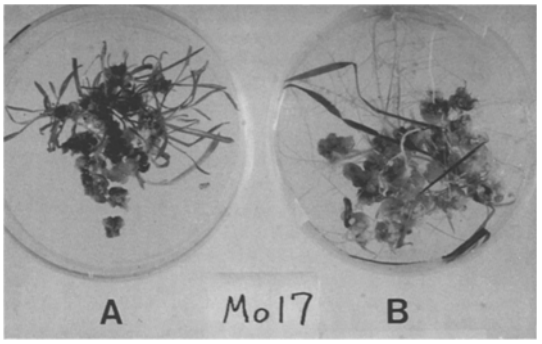

Fig. 1. The effect of 6-benzylaminopurine (6BA) on plant regeneration and root formation from callus of the maize inbred Mo17. To regenerate plants, the callus (0.2 g fresh weight and 28-d old) was grown on H medium with (A) or without (B) 3.5 mg/l 6BA for 21 d. Petri dish is 100 mm wide.

inhibition of root development seems similar to that reported previously by Skoog and Miller (1957) using tobacco and kinetin and Lowe et al. (1985) using maize and kinetin. Consequently, further experiments with 6BA were confined to using the minimum level of 6BA (3.5 mg/l) that substantially increased the number of plants regenerated from maize callus.

Since the 6BA treatment prevented root formation, plantlets exposed to 3.5 mg/l 6BA for 21 d were transferred to H medium containing from 0.2 to 0.8 mg/l (1.0 to 4.2 μM) of 2,4-D, dicamba or NAA for up to 21 d to attempt to induce root formation. Only 0.4 and 0.6 mg/l NAA was observed to stimulate some root formation, but few roots were formed and these did not support continued plant growth (data not included).

Lowe et al. (1985) reported that their regeneration protocol used 10 mg/l kinetin for two to three weeks. To produce roots on their regenerated plantlets, the kinetin treatment was followed by an additional two to three week treatment with 0.1% activated charcoal in medium without growth regulators. The prolonged regeneration time reported by Lowe et al. (1985) stimulated us to look for a faster means of rooting plants exposed to a cytokinin treatment. To find a faster regeneration protocol, we attempted to alter root formation on regenerated plantlets by shortening the time that regenerating tissues were exposed to 6BA.

This effort was prompted by a report by Christianson (1987) working on the transient sensitivity of Convolulus arvensis organogenesis to ribose and sorbitol. In that report, it was demonstrated that organogenesis was inhibited by ribose and sorbitol only when tissue was exposed to the compounds at specific times in a culture cycle. We reasoned that maize tissue might similarly have a transient sensitivity to growth regulators, such as cytokinin, and with the proper application time or length of exposure, whole, rooted plants could be produced from the cytokinin-treated callus tissue.

To determine the effect of length of exposure to 6BA on root formation of regenerated plantlets, Pa91 calli were placed on H medium containing 3.5 mg/l

6BA for 3 to 21 d, following which the calli were transferred to H medium. Calli exposed to 6BA for 3 d produced rooted plants that grew rapidly (Fig. 2). Calli exposed to 6BA for 9 d produced shorter plantlets with fewer roots. Longer exposures to 6BA produced progressively shorter plants with no roots (Fig. 2). Not shown in Fig. 2 are calli exposed to 6BA for 6 d. Root production from these calli was comparable to that of calli exposed to 6BA for 3 d. From these observations, we concluded that a 3 to 6 d exposure to 6BA could maximize production of plants still capable of root formation. Such brief exposure to 6BA caused no visible changes in the calli and plantlet formation was only noticed after the calli had been placed on H medium.

To verify the effectiveness of a 3.5 mg/l 6BA treatment on plant regeneration from maize callus cultures, 21 to 42-d old calli from the maize inbred Pa91 were placed on either H medium or H medium containing 3.5 mg/l 6BA. Since $AgNO_3$ also has been shown to increase plant regeneration from maize callus cultures (Duncan and Widholm, 1987b; Songstad et al., 1988), one half of the calli used in this regeneration experiment were exposed to 200 μM $AgNO_3$ for 21 to 42 d prior to being placed on the regeneration medium. When the mean plant production from calli of all ages are combined, plant production by calli exposed to 6BA was 148% greater than that of control calli exposed to H medium alone (Table 1). However, plant production caused by 6BA was only increased by 113% when both control and 6BA

Table 1. The effect of callus age, $AgNO_3$, and 6-benzylaminopurine (6BA) on plant regeneration from Pa91 maize callus cultures.

Callus Age (days)[b]	Additions to Regeneration Medium	INITIAL GROWTH MEDIUM[a] D medium	D medium plus 200 μM $AgNO_3$
		plants regenerated per gfw of callus[c]	
21	none	10	180
21	3.5 mg/l 6BA	155	335
28	none	260	145
28	3.5 mg/l 6BA	265	285
35	none	80	175
35	3.5 mg/l 6BA	325	345
42	none	60	95
42	3.5 mg/l 6BA	270	300
Ages Combined	none	410	595
	3.5 mg/l 6BA	1015	1265

[a] Callus on initial growth medium for 21 to 42 d followed by either a 3-d exposure to 6BA or no 6BA treatment followed by a minimum of 21 d on regeneration medium (H medium, Duncan et al., 1985).

[b] Callus age refers to length of time on the growth medium prior to the 6BA treatment.

[c] Average of two replicate plates. This is data from one experiment representative of the data obtained from four experiments conducted with Pa91 callus over a nine month period.

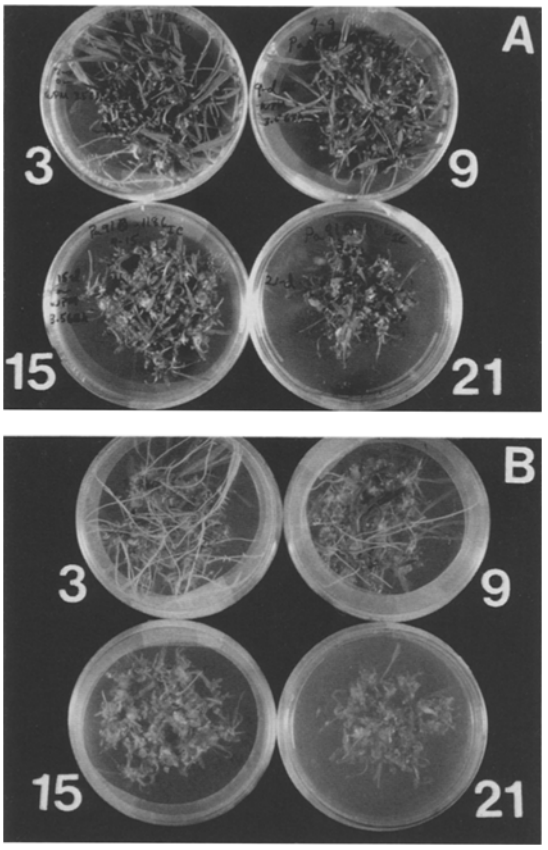

Fig. 2. The effect of exposure time (3, 9, 15 or 21 d) to 6-benzylaminopurine on plant growth (A = top view of plates) and root formation (B = underside view of plates) of plants regenerated from callus of 28-d old maize inbred Pa91. Petri dish is 100 mm wide.

treated calli had first been exposed to $AgNO_3$ (Table 1). These data indicate that a 6BA treatment, when appropriately applied, can dramatically increase plant production from regenerable maize callus cultures.

Calli grown on D medium or D medium plus $AgNO_3$ and also exposed to 6BA had similar levels of plant production (Table 1), which suggests that the 6BA treatment may be stimulating the production of the maximum number of plantlets that a gram of callus from Pa91 can produce. However, calli grown on D medium plus $AgNO_3$ but not exposed to 6BA produced about 45% more plants than control calli not exposed to $AgNO_3$ and 6BA, when the means of all calli age groups are compared (Table 1). These data are consistent with previous reports (Duncan and Widholm, 1986b; Songstad et al., 1988) which showed that $AgNO_3$ can benefit plant regeneration from maize callus cultures. These data also indicate that the slightly lower percentage increase in plant production due to 6BA seen with $AgNO_3$ treated calli, as compared to untreated calli, results from the difference in plant production in the control non-6BA treated calli (Table 1).

It was also noted, that at least for calli not treated with $AgNO_3$ and 6BA, the age of the callus had a dramatic effect on plant regeneration (Table 1) with 28-d old callus producing as many plants from the control treatment lacking 6BA as were

produced from the comparable 6BA treatment. Plant production however, was very low from 21, 35 and 48-d old calli not treated with 6BA. We have observed this in other experiments, as well. The data also show that this effect of age is circumvented by the 6BA treatment and to a lesser extent by $AgNO_3$ (Table 1). Since the effect of callus age can be reduced by 6BA and $AgNO_3$, this age affect may be related to internal concentrations of growth regulators and/or nutrients that may vary throughout the life of callus.

Songstad et al. (1988) showed that ethylene released from maize callus tissue does vary with culture age, peaking in 3 to 10-d old callus and that elevated levels of ethylene do correlate with reduce plant production. Although not measured, the varying ethylene level may influence the number of plants produced by calli of varying ages. Silver nitrate blocks the activity of ethylene (Beyer, 1976). Since $AgNO_3$ was in the test medium during the entire growth period, it would block ethylene activity even as the ethylene concentration changed with culture age. Consequently, the uniform plant regeneration induced by $AgNO_3$ (Table 1) may be due to the suppression of ethylene activity during the entire callus culture cycle prior to the callus being placed on regeneration medium.

The strong effect of 6BA suggests that endogenous IAA or accumulated dicamba may also be involved in the low yield of regenerated plants from the control treatments lacking 6BA (as outlined by Skoog and Miller, 1957). The level of auxins in the maize tissues used in the present study was not measured. However, if they were to fluctuate with callus age then a scenario similar to that just described for ethylene and $AgNO_3$ could be developed for an interaction between 6BA and endogenous auxin levels that may explain the uniform plant regeneration of calli of different ages exposed to 6BA.

In conclusion, an effective regeneration protocol for maize callus cultures grown on D medium consists of subculturing the callus onto H medium containing 3.5 mg/l 6BA for 3 to 6 d and then transferring the callus to H medium for 15 to 21 d. The regenerated plants are then treated as previously described (Duncan et al., 1985). Callus cultures of the self-pollinated inbreds H99 and Mo17 have also been regenerated using this procedure and both showed increased plant production of approximately 129% with the 6BA treatment (data not included). These observations suggest that the 6BA treatment may be useful for many different maize genotypes. These regenerated plants have been grown to maturity and there were no visible differences in appearance and growth between these plants and plants regenerated by the previously used protocol.

This regeneration protocol can dramatically increase the number of rooted plants regenerated from maize tissue cultures. Further protocol modifcations which may increase the amount of callus available for plant regeneration would include the use of 28-d old callus and subculturing the callus from D medium to D medium containing 200 μM $AgNO_3$ prior to plant regeneration.

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