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Full Length Research Paper

Regeneration of green plants from seed-derived callus cultures of *Poa*

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Plants were regenerated from callus derived from entire mature seeds of three *Poa pratensis* cultivars (Kentucky bluegrass, Fylking and Baron) and one Chinese native species, *Poa sphendyLodes* L. Kentucky bluegrass showed the highest regenerating capacity of the four cultivars. Addition of low concentration (0.2 mg/l) of (6-benzyladenine) 6-BA to Murashige and Skoog (MS) callus induction medium containing 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) strongly stimulated seeds inducing shoot-forming callus cultures in Kentucky bluegrass. High percentage of shoots was induced in Shenh and Hilde-brandi (SH) medium containing low concentration (0.1 mg/l) of 2,4-D. Roots were obtained in SH medium with 0.1 mg/l NAA (naphthalene-acetic acid).

Key words: Bluegrass, callus culture, regeneration system, gene transformation.

INTRODUCTION

Bluegrass is a typical cool-season perennial grass with apomictic reproduction (Bashaw et al., 1987). The generation of improved cultivars by conventional breeding has, however, met with major difficulties because of the facultative apomictic mode of reproduction in this species (Kirsten et al., 1993). The future improvement of the important turf and fodder grass relies partly on biotechnological methods such as somatic hybridization and genetic transformation (Kirsten et al., 1993). Efficient tissue culture methods are the prerequisite for the establishment of such biotechnological methods in this species. A number of papers have been published that describe systems for callus culture and plant regeneration in bluegrass using mature embryo and protoplast (Mcdonnell and Conger, 1984; Boyd and Dale, 1986; Kirsten et al., 1993) or immature inflorescences and seeds (Van der Valk et al., 1995; Van der Valk et al., 1989; Van et al., 1991; Chai et al., 2003). Still, the regeneration frequency from seeds is low, and is strongly dependent on the genotype used. Immature inflorescences are suitable explants material (Vander Valk et al., 1989), but they are only available for a short period when the plants start to flower. Seeds are available all year round and are not limited by season. It is essential to find optimum culture conditions in order to increase regeneration frequency.

In the present report, embryogenic callus of bluegrass were induced using mature seeds as explants with different concentrations and combination of auxins and cytokinins on an *in vitro* culture system. The objective of this study was to produce callus cultures with shoot-forming ability from a variety of cultivars for the generation of somaclonal variants and genetic transformation studies.

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Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; **6-BA,** 6-benzylaminopurine; **NAA,** naphthalene-acetic acid; **MS,** Murashige and Skoog; **SH,** Shenh and Hilde-brandi.

MATERIALS AND METHODS

Seeds

Seeds of Yingzhi (*Poa sphendyLodes* L.) (Chinese native) were

Table 1. The effect of basic medium on callus induction frequency.

2,4-D concentration	Basic medium	No. of inoculated seeds	No. of callus	Callus inducing rate ± SD
1.0	MS	89	41	46.1 ± 1.77
	N6	102	26	25.5 ± 0.14
2.0	MS	94	61	64.9 ± 0.50
	N6	126	52	41.3 ± 0.64
3.0	MS	88	56	63.6 ± 1.43
	N6	142	49	34.5 ± 1.62
4.0	MS	90	55	61.1 ± 0.70
	N6	153	51	33.3 ± 0.45

SD: standard deviation.

obtained from Mantou mountain experimental station, in Gansu province. The Kentucky and Fylking seeds were obtained from Australia. And the seeds of Baron are from the Netherlands.

Explant materials

Seeds were put in fresh water for 1 h and dehusked by agitation in 50% (v/v) H_2SO_4 for 30 min and rinsed in running water (3 min) (Torello et at., 1983). They were then surface-sterilized for 30 s in 70% (v/v) ethanol, and washed with sterile distilled water (4~5 times) and then surface-disinfected for 3 min in 0.1% (w/v) $HgCl_2$ (mercuric chloride). They were then washed 8~10 times for 10 min each in sterile distilled water. Fifty seeds were placed in a 9 cm diam. Petri dish containing 25 ml of callus induction medium. Seeds were cultured in the dark at 26°C.

Callus induction and growth

In this experiment, different media and phytohormones were used for callus culture induction. MS (Murashige and Skoog, 1962) and N6 (Zhu Zhiqing et. al., 1975) medium were selected for callus induction, both supplemented with 30 g/l sucrose and 0.7% (w/v) agar, pH 5.8. Different concentrations and combination of auxins [2.4-dichlorophenoxyacetic acid (2,4-D)] and cytokinins [benzylaminopurine (BA)] were added to MS and N6 medium to determine the induction frequency. Incubation was at 26°C in the dark. The material was transferred onto fresh induction medium for further growth. After 8 weeks, callus was scored.

Shoots induction

Compact and/or friable callus were selected for shoots induction with different basal medium (MS and SH) containing 30 g/l sucrose and 0.7% (w/v) agar, and different concentrations and combinations of phytohormones (2,4-D, BA and NAA). After callus were transferred onto regeneration medium, incubation was 26°C /16 h light and 18°C /8 h dark for 60 days. Every two months, new medium was changed.

Roots induction and the tube-plants transfer to soil

About 10 weeks after transfer of callus to regeneration medium plantlets were transferred to roots induction medium (1/2 MS: half of the MS Macro-elements) containing different concentrations of NAA in the 16-h light at 26°C and 8-h dark at 18°C for 30 days. Plantlets with robust roots were selected and cultivated in green house (with enough moisture) for 7-10days without the culture-bottle lid before transfer into the soil. Then plantlets were took out from the callus, and the roots were washed in warm water (23-25°C). The seedlings were transferred to sterile sandy soil, and sufficient water was supplied every day.

RESULTS

Callus induction and growth

The average germination frequency of seeds in all experiments throughout this study was 90%. Because only germinating seeds produced callus, quantitative determinations of the morphogenetic response of callus cultures were related to germinating seeds (Van der Valk et al., 1989; Ruis et al., 1995).

Effects of different medium on callus induction

As shown in Table 1, the callus inducing frequency of Kentucky was different on MS and N6 medium containing different concentrations of 2,4-D. The inducing rates of MS medium was significantly higher than those of N6 medium (P<0.01). When MS medium was supplemented with 2.0 mg/L 2,4-D, the highest inducing rate was obtained (64.9%).

Effects of different concentrations and combination of phytohormones on callus induction

The effects of different concentrations of 2,4-D in MS medium were studied using four cultivars (Kentucky, Baron, Fylking and Yingzhi) (Figure 1). The induction rate of Kentucky and Baron was significantly higher than that of Yingzhi and Fylking (P<0.01). Compact and solid type of callus in Kentucky was observed. However most callus

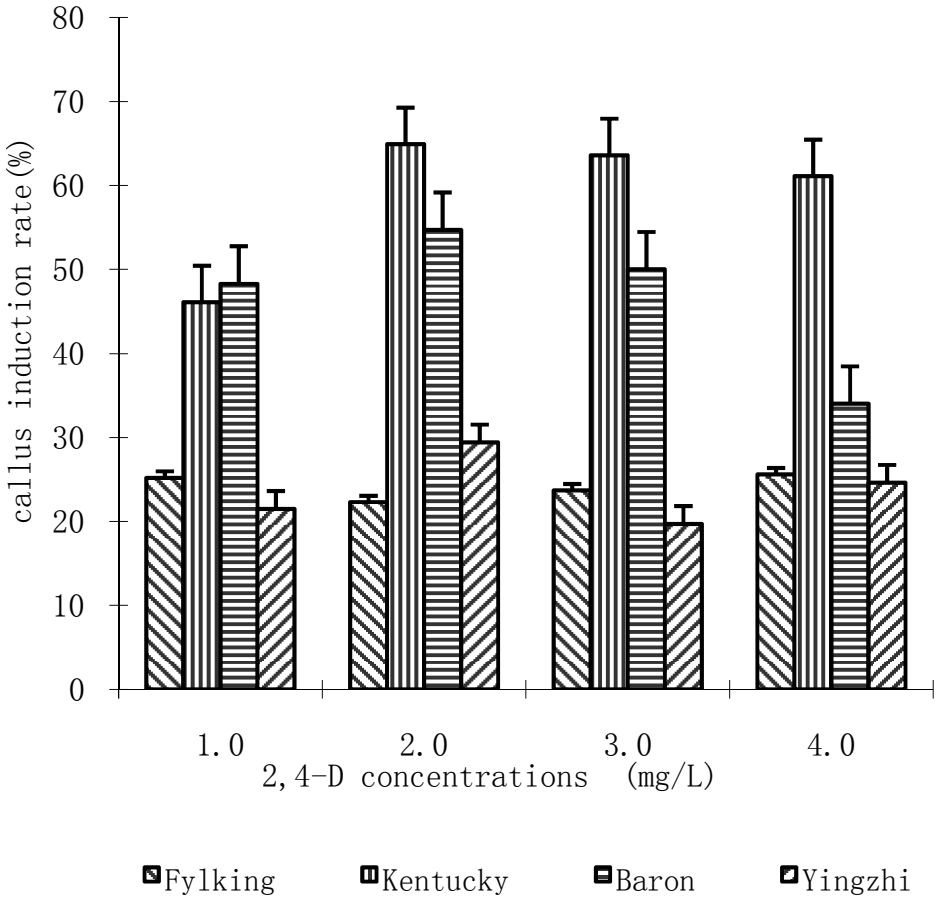

Figure 1. The effect of 2,4-D concentrations on different cultivars of bluegrass' callus induction. Vertical line on each bar represents standard error of the mean.

from Baron were soft and watery. It had the highest callus induction frequency (64.9%), which is higher than all other species, when the concentration of 2,4-D was up to 2.0 mg/l.

Effects of different concentration's combination of 2,4-D and 6-BA on callus induction

Different combinations of auxins (2,4-D) and cytokinins (BA) in MS medium with respect to their callus induction capacity in mature seeds base explants (Figure 2). Inclusion of low concentrations (0.1-0.3 mg/l) of BA in the callus induction medium strongly promoted the frequency of callus induction and the development of compact and embryogenic callus in four cultivars. The production of soft and watery callus was reduced on BA-containing medium. Callus on medium containing BA tended to turn compact and brown more easily than on medium devoid of BA. Browning of callus was more pronounced in Kentucky than the other three cultivars, and the results were significant (P<0.01). The highest induction rate (78.5%) was obtained when 3 mg/l 2,4-D and 0.1 mg/l 6-BA were supplemented for Kentucky (Figure 2). Compared with when only 2,4-D was used, 2,4-D supplemented with BA were beneficial for callus induction.

Shoots induction

Effects of different medium on shoots induction

Two kinds of medium containing 0.1 mg/l 2,4-D were measured in this research (SH and MS) (Table 2). The mean number of Kentucky differentiated shoots on SH medium (8.1) were significantly higher those that on MS medium (3.6) (P<0.05).

Effects of different concentrations of 2,4-D on callus differentiation

As shown in Figure 3, one cultivar, Kentucky was measured on SH medium. When the concentration of 2, 4-D

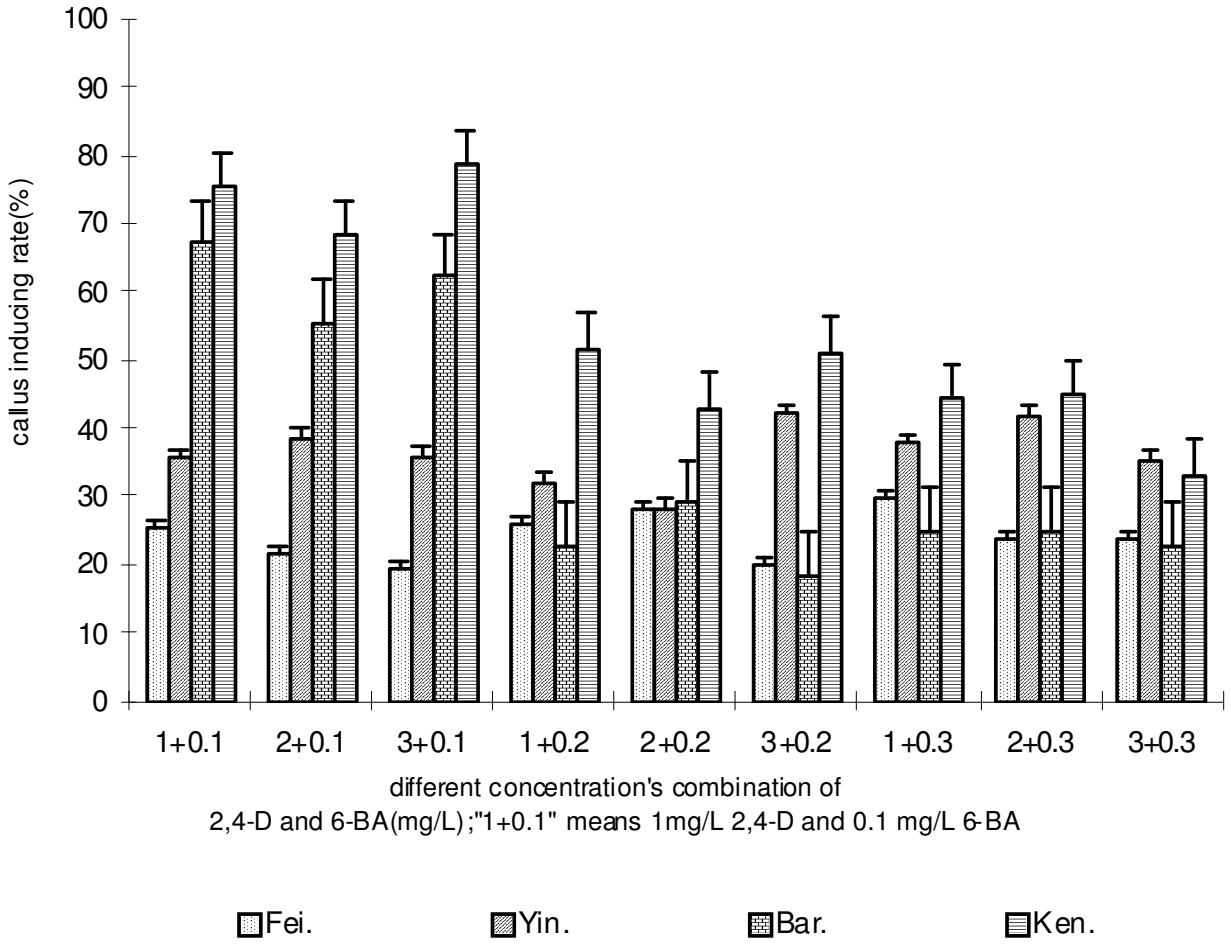

Figure 2. The effect of different combination of 2,4-D and 6-BA concentration on callus induction. Vertical line on each bar represents standard error of the mean.

Table 2. The effect of basic medium on callus differentiation.

Basic medium	Total number of callus	Total number of shoots	Mean number of differentiated shoots ± SD
SH	61	494	8.1 ± 0.36a
MS	52	187	3.6 ± 1.25b

SD: standard deviation.

was 0.1mg/l, the mean number of differentiated shoots were up to the highest number (8.1).

Roots induction and the tube-plants transfer to soil

Effects of different medium on roots induction

1/2 MS and WPM medium containing 1.0 mg/l NAA were applied for Kentucky root induction (Table 3). 1/2 MS medium was significant better than WPM for roots induction (P<0.05).

Effects of different concentration of NAA on rooting rate

For Kentucky, rooting rate was up to the highest when the concentration of NAA was 1.0 mg/l on 1/2 MS medium (Figure 4). And the induced roots were robust and numerous.

Tube-plants transfer

Healthy and robust tube-plants were selected to transfer

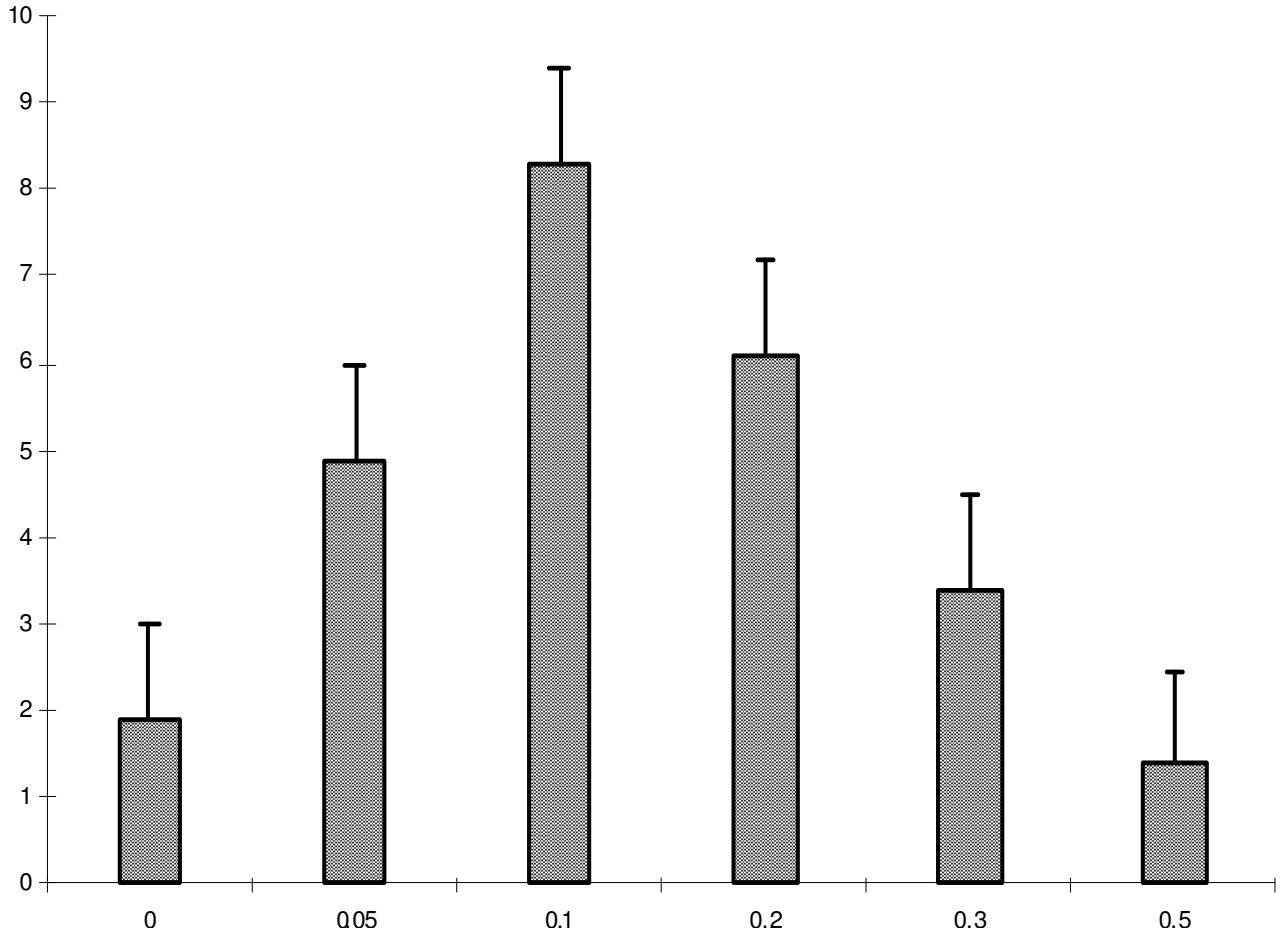

Figure 3. The effect of 2,4-D concentration on callus differentiation. Vertical line on each bar represents standard error of the mean.

Table 3. The effect of different basic medium on rooting frequency.

Basic medium	NAA concentration (mg/l)	No. of transferring shoots	No. of shoots with root	Rooting rate ±SD (%)
1/2MS	1.0	34	28	82.4 ± 2.85a
WPM	1.0	41	21	51.6 ± 2.83b

SD, standard deviation; NAA, naphthalene-acetic acid.

to the soil (Figures 5a-i). The survival rate was up to 90%.

DISCUSSION

Bluegrass is an important forage and turf grass with facultative apomictic mode of reproduction and is, in general, recalcitrant in tissue culture. Moreover, the information regarding the effects of different factors on blcuegrass tissue culture is lacking. The results presented show that Kentucky was excellent for tissue culture from seed-derived callus culture in four cultivars. Inclusion of a low concentration of BA in the callus induction medium resulted in the formation of a very compact, embryogenic structure on callus with a relatively high percentage.

Tissue culture for grass family is obviously more difficult than for legume and the disparity is significant (Xu, 2001). Mcdonnell and Conger (1984) reported the significant difference of five bluegrass cultivars（Blacksburg, Majestic, Eclipse, Ram I, Glade, South Dakota）for tissue culture from seed-derived callus. The results was the same with the reports of van der Valk et al. (1989), while Zhu Genfa and Yu, (1994) reported there was no significant difference

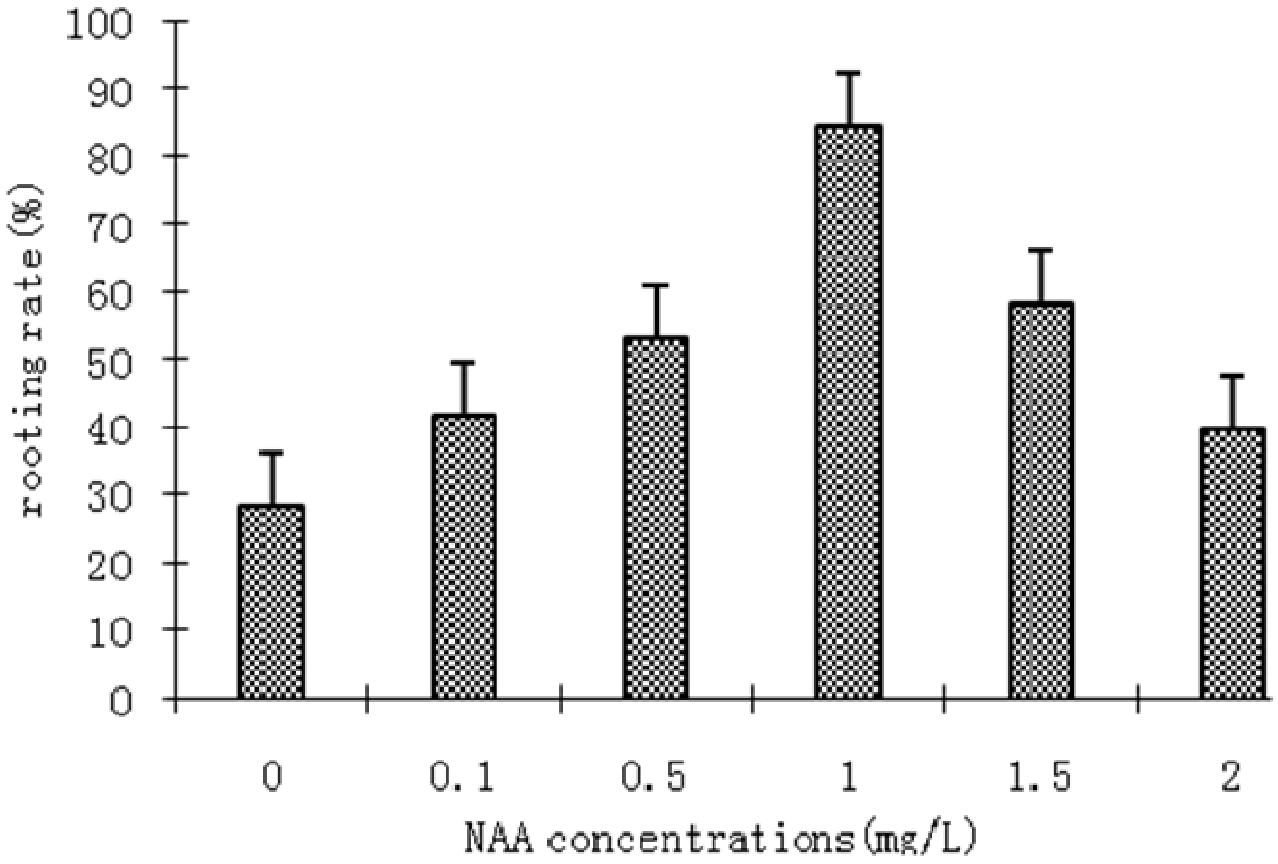

Figure 4. The effect of different NAA concentrations on bluegrass' rooting frequency. Vertical line on each bar represents stand error of the mean.

between two bluegrass cultivars (Wabash and Challenger) for regeneration from young inflorescence as explants. The present report indicated that Kentucky was the optimum cultivar for regeneration from seed-derived callus of four species.

Auxin (2,4-D) is known to enhance callus-forming ability and is mostly used in the callus induction medium in plant tissue culture. In the present study, the callus inducing rate was very low when only 2,4-D was applied. This was different with the report of Ma ZH et al. (1999). Perhaps different cultivars have different reactions to 2,4-D. A certain ratio of auxin and cytokinin in the callus induction and subculture medium often improves culture quality. In this experiment, the percentage of seeds producing shoot-forming callus was significantly higher on BA-containing media as compared to media devoid of BA. Improved regeneration by the addition of BA to callus induction medium was previously reported in bluegrass (van der Valk et al., 1989). The need for added cytokinins for the production of regenerable cultures could be explaned by genotypes having defferent levels of endogenous cytokinins (Shyamala and Roberta, 1990).

Some reports have shown that in bluegrass the morphogenic response of immature inflorescence segments is much higher than that of germinating mature seeds (Van der Valk et al., 1989; McDonnell and Conger, 1984). Although immature inflorescence is better than mature seeds for bluegrass regeneration, the application is restricted by the season. Mature seeds are easy to obtain and are not limited by different seasons and plant developing period. It is indispensable to find optimum conditions for bluegrass culture from seed-derived callus culture. Our results suggest that for bluegrass regeneration it is important to select varieties with a high callus induction rate. The regeneration frequency from seed-derived callus is still low and limited to one cultivar. More work should be done to improve bluegrass regeneration frequency. The results however imply that plants based on *in vitro* culture techniques will be an important and useful tool in future breeding programmes for improved cultivars of bluegrass.

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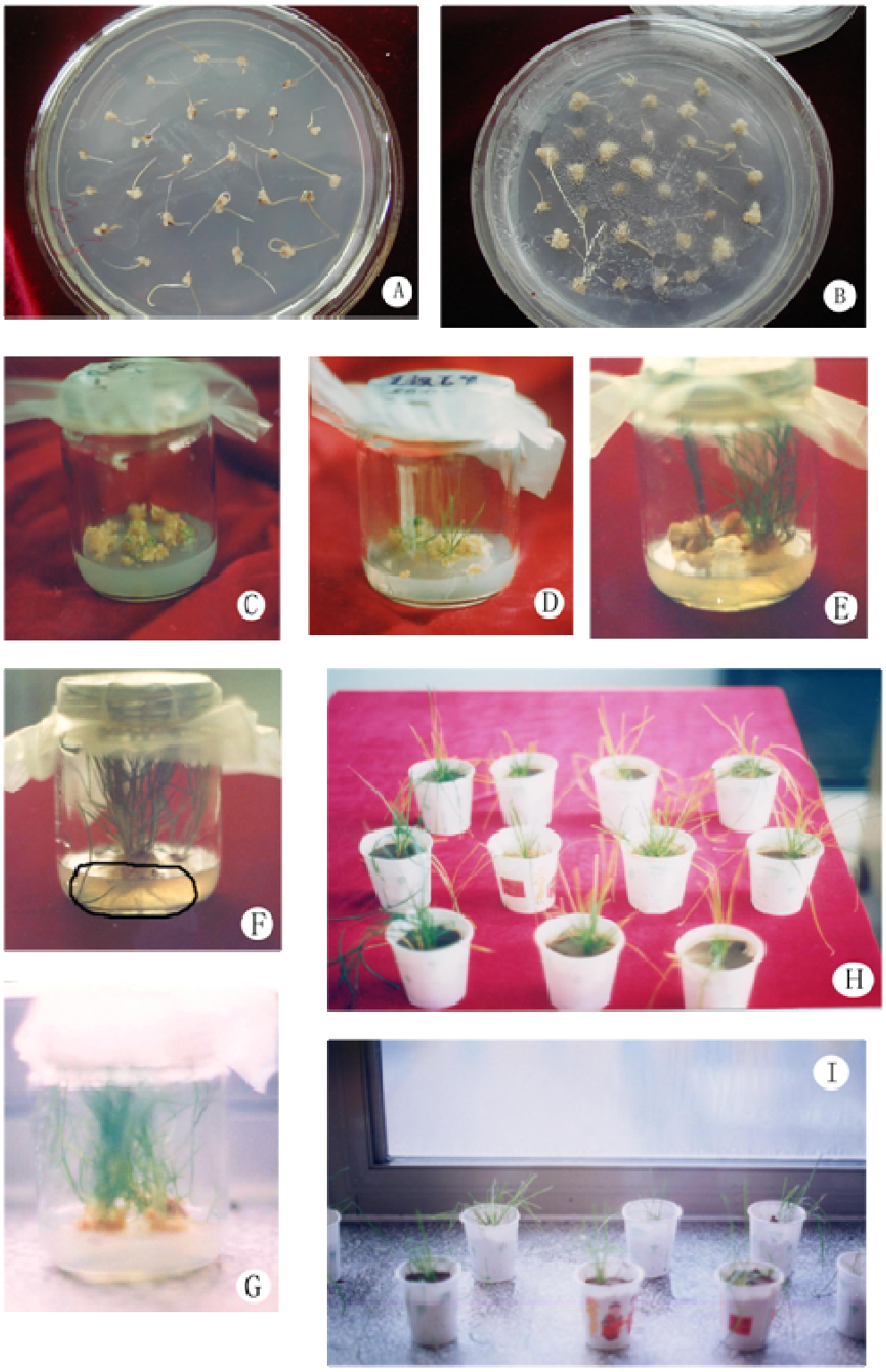

Figures 5. Callus induction, plant regeneration and tube-plants transfer testing. a) Callus formation from mature seeds, two weeks after inoculation. b) Callus development 4 weeks after mature seeds on callus induction medium. c) Formation of bud, 3 weeks after induction from callus. d) Plant regeneration of exclusively green plantlets, 5 weeks after induction from callus. e) Healthy and robust tube-plants, 7 weeks after induction from callus. f) roots formation 3 weeks after plantlets induced on roots induction medium. g) tube-plants acclimated in green house. h) tube-plants transfer to the soil. i) plants obtained from seed-derived callus in field test, 2 weeks after transfer to the soil.

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