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Re-evaluation of Mature Seed-derived Callusing and Regeneration Potential of Nine Orchardgrass (*Dactylis golomerata* L.) Cultivars

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Abstract

A suitable callus induction and efficient regeneration protocol for orchardgrass (*Dactylis golomerata* L.) was developed. It consisted of 3 mg/l 2,4-D + 0.1 mg/l BA + 1 g/l CH + 300 mg/l L-proline + 40 mg/l L-cysteine + 30 g/l sucrose in MS showed the highest percentage of callus induction. Maltose exhibited better in regeneration than other types of carbon sources. Highest (71%) regeneration was obtained from N_6 medium containing 1 mg/l 2,4-D + 3 mg/l BA + 1 g/l CH + 300 mg/l L-proline + 40 mg/l L-cysteine + 30 g/l maltose. Among the nine cultivars of orchardgrass (*Dactylis golomerata* L.), genotypic variation was observed in both callus induction and regeneration. Overall callus induction and regeneration rates were 23 - 73 and 17 - 71%, respectively.

Introduction

As a forage crop, orchardgrass (*Dactylis golomerata* L.) provides an excellent high quality feed for most classes of livestocks and regarded as a versatile grass species that can be used for pasture, hay, green chop or silage (Miller 1984) but rapid decline in palatability and quality of mature orchardgrass is the major deterrent to its uses. A report on orchardgrass comparing with tall fescue revealed that livestock grazing of orchardgrass showed higher weight gain than that of the tall fescue. Therefore, orchardgrass is found to be better than other types of grasses. Due to self-incompatibility in orchardgrass it is a very difficult and time consuming to obtain a new variety through conventional breeding (Gray et al. 1984). Therefore, use of biotechnological tools including *in vitro*

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culture/or genetic transformation may be considered for the improvement of quality and enhancement of resistance to different abiotic or biotic stresses of this grass species.

Establishment of highly efficient and reproducible regeneration system would greatly influence the efforts of improvement of this grass species through gene transfer technology. Plant regeneration of orchardgrass through organogenesis or by somatic embryogenisis has been reported earlier from intact caryopses (Conger and Carabia 1978), leaf derived mesophyll cells (Hanning and Conger 1982, Conger et al. 1983), anther culture (Songstad and Conger 1988) or by suspension culture (Gray et al. 1984, Gray and Conger 1885, Trigiano and Conger 1987, Horn et al. 1988). A few reports on plant regeneration and subsequent regeneration of transgenic plants have been obtained of orchardgrass through mature seed derive callus (Cho et al. 2001). However, to initiate *in vitro* cultures in Gramineae mature seeds is widely used as a source of explants (Conger 1981, Jiang et al. 1998, Ha et al. 2001, Wang and Ge 2005, Dong and Qu 2005). Nowadays, mature or dry seeds are considered an important source of explants, and are extensively used for plant regeneration via callus culture or somatic embryogenesis. We therefore, made an attempt to develop an efficient and reproducible regeneration system from mature seed-derived callus of orchardgrass for genetic transformation. In order to optimize the callus induction and the subsequent regeneration from seed-derived callus, a broad spectrum of cultural conditions was examined and appropriate conditions were selected through stepwise optimization.

Materials and Methods

Mature seeds of nine cultivars of orchardgrass (*Dactylis golomerata* L.), namely, Hapsung 2, Hapsung 21, 93E, Amba, Ambassador, Frontier, Frode, Potomac and Roughrider were collected. Seeds of Hapsung 2, Hapsung 21, 93E, Amba, Ambassador, Frontier and Potomac were supplied by the National Livestock Research Institute (Suwon, Korea) and Roughrider was purchased from Duck-Chang Agri-Bioscience Company Ltd. (Sung Nam, Korea). Cultivar Roughrider was used as the initial model for optimization of tissue cultural conditions. Approximately 5 g of mature seeds of the selected cultivars were dehusked manually and rinsed with 70% ethanol for 1 - 2 min. After rinsing with distilled water three times seeds were surface-sterilized by immersion in a solution of 5% (w/v) sodium hypochlorite (Junsei, Japan) for 30 min. Two drops of Tween-20 (Junsei, Japan) were added. To remove the surfactants, sterilized seeds were rinsed three times with sterile deionized-distilled water and blotted on to a sterile Whatman filter paper.

Embryogenic callus induction and subsequent plant regeneration: Approximately, 100 sterilized dry seeds were placed on MS containing 3 mg/l 2,4-D, 0.1 mg/l BA, 1 g/l CH, 300 mg/l L-proline and 40 mg/l of L-cysteine for primary callus induction. Sucrose (30 g/l) was added. Twenty seeds were transferred on each Petri dish (87 × 15 mm) containing 30 ml of callus induction medium. The cultures were transferred to controlled growth chamber at 24 ± 2°C under continuous dark. After four weeks callus was removed manually from the germinating shoots and roots, and sub-cultured on the same medium and conditions for a couple of weeks.

For regeneration, two weeks old compact, nodular embryogenic callus were plated on to N_6 (Chu et al. 1975) basal medium containing 1 mg/l 2,4-D, 3 mg/l BA, 1 g/l CH, 300 mg/l L-proline and 40 mg/l of L-cysteine and kept under fluorescent light (80 μE m^{-2} s^{-1}) at 16-h photoperiod. Maltose (30 g/l) was added to the regeneration medium. Shoots initiated within four weeks after plating. For all experiments, the medium was solidified with 2 g/l Gelrite (Duchefa Biochemie, Netherlands), and the pH was adjusted to 5.7 before autoclaving. After four weeks, regenerated shoots (1 - 2 cm) were transferred to glass jars for further development.

Rooting and acclimation of regenerated plants: For the development of a strong root system, regenerated shoots (3 - 4 cm) were further transferred to the rooting medium consisting of half-strength of MS with 20 g/l sucrose. Cultures for rooting were kept under fluorescent light (80 μE/m^{-2}/s^{-1}) at 24 ± 2°C and 16-h photoperiod for a couple of weeks. Rooting was observed within two weeks. Plantlets with well developed roots were removed carefully from the agar gel, and the medium was rinsed off the roots with sterile water. The plantlets were placed in plastic pots, and the roots were covered with the Horticulture Nursery Media (Biomedia, Korea) and grown in growth chamber at 22°C day/20°C night cycle under a 16-h photoperiod. The light intensity was 350 μE/m^{-2}/s^{-1}.

Factors evaluated: To optimize suitable growth regulators and their optimum concentration and combinations for seed-derived callus induction, different kinds of auxins (2, 4-D, Dicamba, NAA, IAA) alone or in a combination with BA were used in MS containing 30 g/l sucrose. Types and levels of growth regulators were recorded. To elucidate the appropriate condition for plant regeneration two auxins (2,4-D and NAA) and two cytokinins combinations were used at various concentrations. Different kinds of additives such as CH, L-proline, $AgNO_3$ or L-cysteine were added individually or in combinations at various concentrations of growth regulators to find out the best combination to support growth and differentiation. To determine the role of carbon sources for callus induction and plant regeneration a wide range of saccharides: glucose, sucrose, maltose, lactose and mannitol at a concentration of 30 g/l were tested

with an optimum level of growth regulator- and additive-fortified selected basal media. Three different kinds of basal medium: MS, N_6 and SH (Schenk and Hildebrandt 1972) were investigated to determine the effect of each basal medium on callus induction and plant regeneration.

Results and Discussion

Effect of growth regulators to callus induction: Callus induction frequency was measured as percentage of seeds that produced callus. To examine the effect of growth regulators on callus induction, first, various kinds of auxins (2,4-D, Dicamba, NAA and IAA) were used singly at different concentrations (1 to 5 mg/l) (Table 1) and, second, optimum concentration of auxins was used in

Table 1. Effect of growth regulators on plant regeneration[a].

Growth regulators (mg/l)		Shoot regeneration frequency (%)[b,c]
2,4-D	BA	
1	1	23 ± 3.60
	3	38.33 ± 3.51
	5	26.66 ± 4.04
2	1	15.33 ± 3.21
	3	22.33 ± 3.05
	5	18.66 ± 3.21
2,4-D	Kinetin	
1	1	24 ± 4.58
	3	31.66 ± 3.05
	5	28 ± 4.51
2	1	14 ± 2.64
	3	20.66 ± 3.51
	5	21 ± 3.6
NAA	BA	
1	1	11.33 ± 3.51
	3	24.33 ± 4.5
	5	31.33 ± 4.04
2	1	9.33 ± 3.21
	3	13.33 ± 2.51
	5	15 ± 2.64
NAA	Kinetin	
1	1	12 ± 3.6
	3	23.33 ± 3.21
	5	34 ± 3.6
2	1	10.33 ± 2.51
	3	13.66 ± 3.78
	5	19 ± 4

[a]Embryogenic callus was maintained on MS containing 30 g/l of sucrose with different concentrations of growth regulators in multiple combinations. [b]Shoot regeneration frequency as estimated in terms of total number of shoots per 100 callus pieces used in each treatment. [c]Data represent the mean values of three replicated experiments ± Sd. Values were significantly different (ANOVA, $p < 0.05$).

combination with BA (0.1 to 10 mg/l) (Fig. 1). The callus induction frequency varied from 46 to 19%. Among all auxins, both 2,4-D and Dicamba at a concentration of 3 mg/l each produced 43 and 42% callus, respectively (Fig. 2). Addition of BA at different concentrations with the optimum concentration of 2,4-D or Dicamba increased callusing frequency from 43 to 46% but reduced the callus weight (Fig. 3). Callus weight and morphogenic responses varied according to growth regulators and their, concentrations and combinations 2,4-D or Dicamba along with BA did not result in any significant

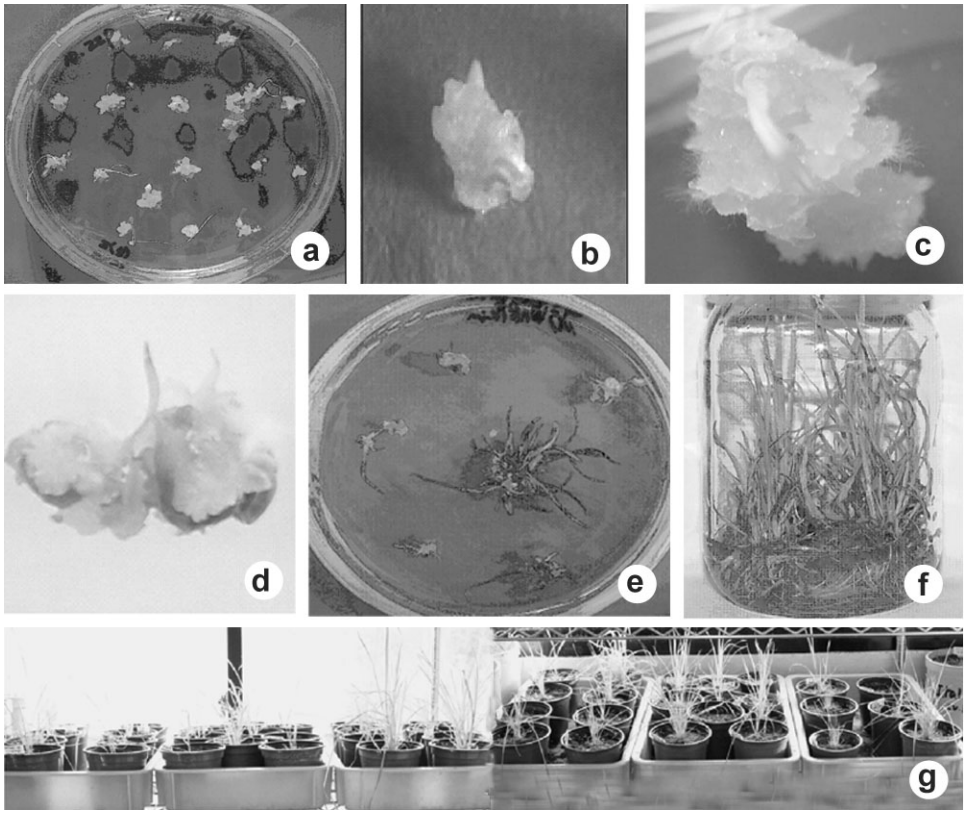

Fig. 1. Primary callus induction, secondary growth of callus and plant regeneration from dehusked mature seeds of cv. Roughrider. a. Calli appeared from the mature seeds. b. Germinating shoots and roots were removed form the calli. c. Compact nodular embryogenic calli. d. Regeneration of shoots four weeks after plating. e. Regenerated plants were subcultured. f. Regenerated plants in the rooting medium and g. Plantlets growing in the greenhouse.

difference in callus induction frequency. However, Dicamba showed slightly higher weight of callus than 2,4-D. Effect of 2,4-D and Dicamba on callus induction frequency was not significant. We, therefore, used 2,4-D because of its low cost than Dicamba. For subsequent experiments 3 mg/l of 2,4-D with 0.1 mg/l of BA as two supplements were added to MS medium.

Effect of growth regulators on plant regeneration: Green shoots were observed within 2 - 3 weeks of culture. Among all concentrations and combinations of auxins and cytokinins, MS containing 1 mg/l of 2,4-D, 3 mg/l of BA and 30 g/l of sucrose showed highest percentage (38) of shoot regeneration as well as early shoot induction (Table 1, Fig. 1). Therefore, this combination was selected for further experiments.

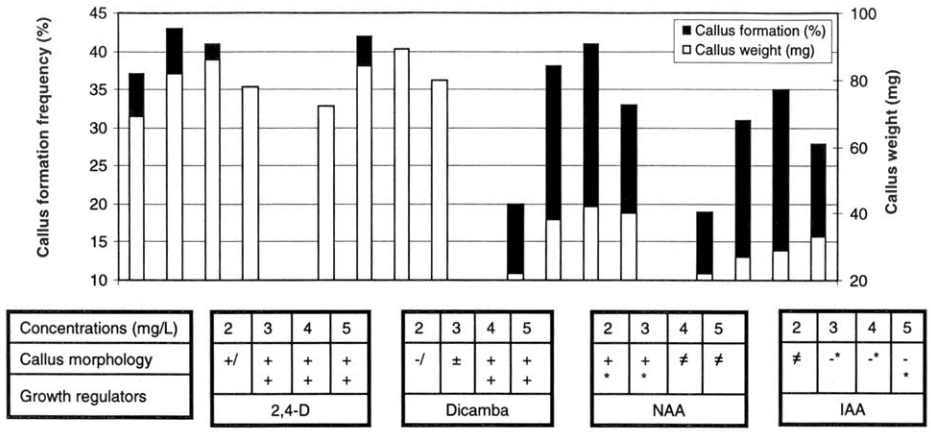

Fig. 2. Effect of auxin on callus induction frequency of cv. Roughrider. Callus morphology: Compact light green (+/), compact green (++), friable light green (–/), friable green (±), compact yellow (+*), friable yellow (–*), friable whitish (≠). Dehusked mature seeds were cultured on MS containing 30 g/l sucrose with supplements of auxins.

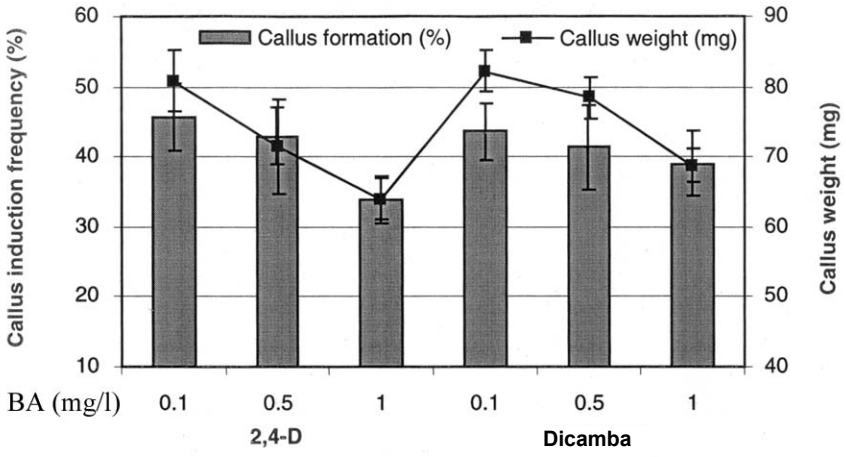

Fig. 3. Combination of auxins with cytokinin to callus induction from mature seeds of cv. Roughrider. Dehusked mature seeds were cultured on MS containing 30 g/l sucrose, 3 mg/l 2,4-D or Dicamba and afore-mentioned concentrations of BA. The values are means of ± Sd; all experiments were replicated three times. The values are significantly different (ANOVA, $p < 0.05$).

Effects of additives on callus induction and plant regeneration: Effects of different additives to embryogenic callus formation and plantlet regeneration were studied. Various kinds of plant growth additives, such as casein hydrolysate, L-proline, L-cysteine and $AgNO_3$ at various concentrations were used either individually or in combinations (Table 2) added to callus inducing and regeneration medium in order to determine their effects on callus induction and plant regeneration. Additions of supplements at different concentrations and combinations significantly improved the proliferation rate of callus from 46 to 73% and regeneration ability from 38 to 61%. Single additive at different concentrations slightly raised both callus induction and regeneration frequency. On the other hand, combinations of optimum level of additives for callus induction and regeneration yielded far better results compared to the effects of single additives; i.e., the callus induction and plant regeneration frequency significantly increased from 46 to 73% and 38 to 61%, respectively (Table 2).

Table 2. Effect of different growth additives on callus induction and plant regeneration[a].

Name of the additive (s)	Concentrations	Callus induction frequency (%)[b]	Shoot regeneration frequency (%)[c]
Casein hydrolysate	1 g/l	50 ± 3.6	50.33 ± 3.05
	2 g/l	47.66 ± 4.04	45 ± 3.6
	3 g/l	47.33 ± 4.5	44 ± 3.6
L-proline	200 mg/l	46.66 ± 45	41.66 ± 4.16
	300 mg/l	49 ± 4.58	43.33 ± 4.5
	400 mg/l	47.66 ± 4.04	39 ± 5.29
Cysteine	20 mg/l	47 +/- 3.6	41.66 +/- 3.78
	40 mg/l	50.66 ± 4.04	46.66 ± 2.51
	60 mg/l	48.66 ± 3.05	47 ± 2
$AgNO_3$	5 mg/l	50.33 ± 2.51	50 ± 2.64
	10 mg/l	53.33 ± 2.08	55.66 ± 2.51
	15 mg/l	52 ± 3	53.33 ± 3.05
CH+ L-proline + cysteine + $AgNO_3$	1 g/l + 300 mg/l + 40 mg/l + 10 mg/l	73 ± 2.6	61.33 ± 3.51

[a]There were three replications for each treatment with 100 seeds per treatment; data represent the mean values ± Sd. [b]Seeds were cultured as described in Fig. 2 with additives. [c]Calli were transferred on to the regeneration medium as described in Table 1 with additives.

Effect of carbon sources to callus induction and regeneration: Various types of carbon sources such as monosaccharides (glucose, mannitol and sorbitol) and disaccharides (sucrose, maltose and lactose) were examined to evaluate their respective effects on callus induction and subsequent plant regeneration

frequency. Significant differences were found among carbon sources tested to callus formation and subsequent regeneration. Disaccharides performed better than monosaccharides. However, glucose performance was much higher than lactose to callus formation and subsequent regeneration. The highest 73%; i.e. seeds induced callus when 30 g/l of sucrose was added as a carbon source. However, in case of shoot regeneration maltose showed superior result than the other carbon sources. The highest, 68% callus showed shoots regeneration when they were cultured using 30 g/l maltose in the optimized shoot regeneration medium. In contrast, 61% shoot regeneration were observed in optimized shoot regeneration medium containing 30 g/l of sucrose (Fig. 4). Sucrose and maltose at a concentration of 30 g/l were therefore selected as a superior carbon source for callus induction and plant regeneration, respectively.

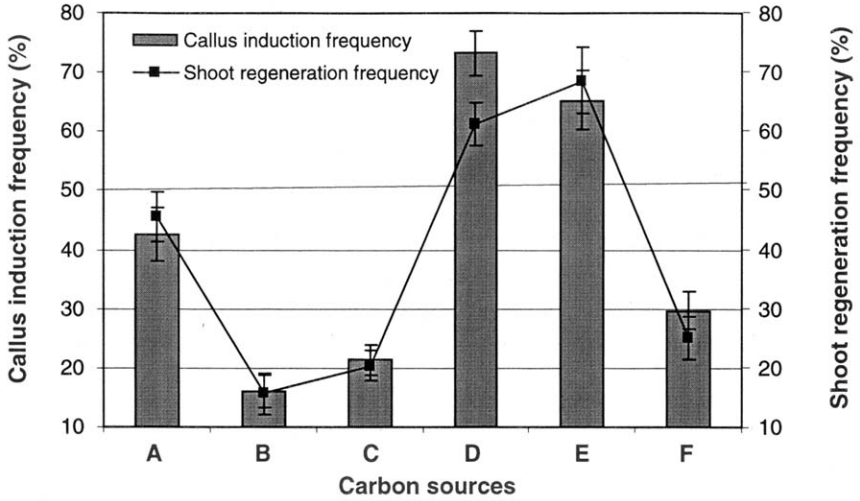

Fig. 4. Effect of various carbon sources on callus induction and shoot regeneration. The cultural condition for callus induction and subsequent regeneration are the same as described in Table 2 except carbon source. A, glucose; B, mannitol; C, sorbitol; D, sucrose; E, maltose; F, lactose. The values are means of ± Sd; all experiments were replicated three times. Values were significantly different (ANOVA, $p < 0.01$).

Effect of basal medium on callus induction and plant regeneration: Three different kinds of basal mediums (MS, N_6 and SH) were used to investigate their effects on callus induction and regeneration. The previous optimized callus induction and regeneration conditions were applied in the three basal media. Significant differences to callus induction and plant regeneration frequency were observed. The highest, 73.33 % seeds callused when they were cultured on MS under previously described optimized conditions of growth regulators, additives and carbon source. Whereas N_6 basal medium appeared to the best medium for

maximum (71.66%) shoot regeneration, in SH medium both volume of callusing and plant regeneration frequency were lower compared to other media (Fig. 5).

Genotypic variation to callus induction and plantlets regeneration: Considerable genotypic variation was observed in both callus induction and plant regeneration (Fig. 6). Callus induction and shoot regeneration frequency ranged from 23 to 73% and 17 to 71.66%, respectively. Cultivar Roughrider showed relatively higher callus induction (73.33%) and regeneration (71.66%) than the remaining seven cultivars. In contrast, in cultivar Hapsung 21 callusing was low, only 23%; and shoot regeneration frequency was even lower (only 17.33%). Callusing induction and regeneration frequency were more than 50% in Frode and Frontier cultivars.

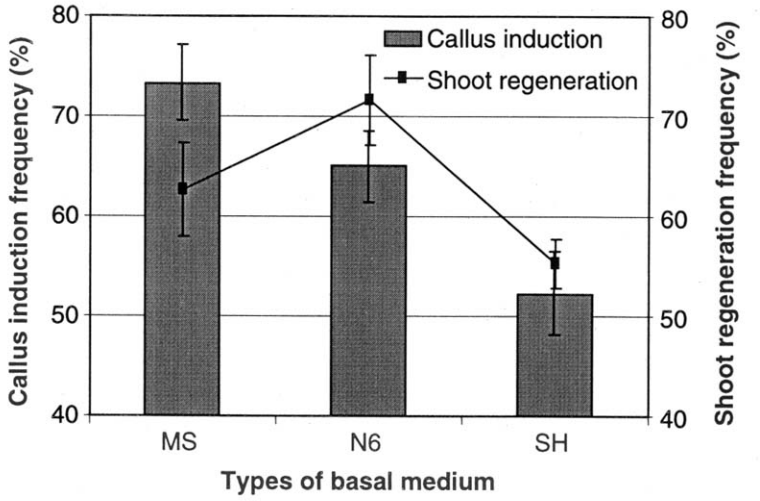

Fig. 5. Effects of basal medium on callus induction and shoot regeneration. For callus induction, each basal medium was fortified with 3 mg/l 2,4-D + 0.1 mg/l BA + casein hydrolysate 1 g/l + L-proline 300 mg/l + L-cysteine 40 mg/l + $AgNO_3$ 10 mg/l the carbon source being 30 g/l of sucrose; for plant regeneration the supplements were: 1 mg/l 2,4-D, 3 mg/L BA, 30 g/l of maltose plus the same additives as used for callus induction. The values are means of ± Sd; all experiments were replicated three times. According to ANOVA test values are significantly different at $p < 0.05$.

Extensive literature is not available on callusing of mature seeds and subsequent regeneration. The present study reports the results of a wide range of callus induction and subsequent plant regeneration. Media optimized for mature seeds of nine orchardgrass cultivars. In previous studies on orchardgrass, mature seeds were used as a source of explants for callusing and subsequent plant regeneration or in transformation protocols (Cho et al. 2001, Lee et al. 2006) or in other grass species (Griffin and Dibble 1995; Bai and Qu 2000; Dong and Qu 2005; Wang and Ge 2005; Lee et al. 2007).

Among all auxins, 2,4-D has been widely used for callus induction for the plants of the Gramineae family (Sirkka and Immonen 1993; Bai and Qu 2000; Chaudhury and Qu, 2000). In our studies callus induction frequency was examined by single use of different kinds of auxins at various concentrations or in combinations with different cytokinins. Single use of 2,4-D or 3 mg/l Dicamba in MS was equally effective in callus induction compared to other auxins. Inclusion of a lower concentration of cytokinin with auxin was found to increase the callus induction frequency, callus quality and its regeneration capability (Chaudhury and Qu 2000, Dahleen and Bregitzer 2002, Huang and Wei 2004). In the present study, we also examined BA at 0.1, 0.5 and 1.0 mg/l along with 3 mg/l of 2,4-D or Dicamba. Addition of 0.1 mg/l BA in combination with an auxin

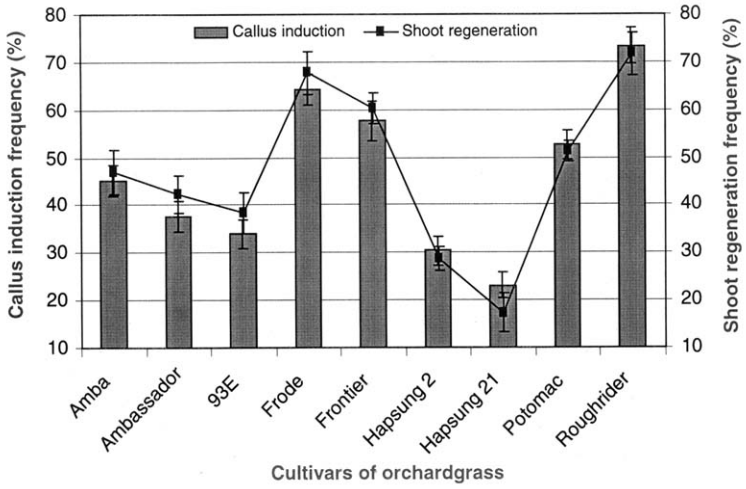

Fig. 6. Callus induction and subsequent shoot regeneration frequency from mature seeds in nine different cultivars of orchardgrass. Callus induction and shoot regeneration were initiated on MS and N_6, respectively. Concentrations and combination of growth regulators, additives and carbon sources are the same as shown in Fig. 5. The values are means of ± Sd; all experiments were replicated three times. Values were significantly different (ANOVA, $p < 0.01$).

increases the callus induction frequency than by the single use of 2,4-D or Dicamba. However, a combination of 2,4-D and BA was found to improve slightly the frequency of callus induction compared to Dicamba. Our findings also support earlier reports on leaf segments-derived somatic embryogenesis of orchardgrass (Hanning and Conger 1982), where 2,4-D gave equivalent or superior results than Dicamba. Among all combinations and concentrations, 1 mg/l of 2,4-D + 3 mg/l of BA fortified MS medium was found to be superior than that of auxin and cytokinin combination. A higher concentration of

cytokinin in combination with a low level of auxin was found to be superior for plant regeneration via callus (Prakash et al. 1999, Yang et al. 1999, Tiidema and Truve 2004).

Yang and Hoefman (1984) and Finkelstein et al. (1988) reported that different inhibitory substances such as ethylene (C_2H_4) are formed during various stages of tissue culture experiments. Formation of such chemicals slows down both callus proliferation and regeneration process (Chi et al. 1991, Pua and Chi 1993). On the other hand, some chemical compounds, when added as a supplement to the basic medium such as casein hydrolysate were found to produce a stimulatory effect on callus induction, proliferation, somatic embryogenesis including regeneration (Gray et al. 1984, Gray and Conger 1985, Hossain et al. 1993, Prakash et al. 1999), so also proline (Yang et al. 1999, Lee et al. 2003) or $AgNO_3$ (Vain et al. 1989, Adkins et al. 1993, Fei et al. 2000) or cysteine (Enriquez-Oberegon et al. 1999). Addition of additives increases the callus induction from 46 to 53% and regeneration frequency from 38 to 55% as against the use of an optimum growth regulator. Similar to other reports (Conger et al. 1983, Gray et al. 1984) inclusion of CH to the medium did not show any significant effect on callusing; however, to obtain somatic embryogenesis (Gray et al. 1984; Gray and Conger 1985), and shoot differentiation CH was found to be essential (Prakash et al. 1999). Gray and Conger (1985) reported that proline had no effect on callus culture of orchardgrass, while some other Gramineae species showed higher callus induction (Yang et al. 1999) without any significant effect on regeneration. Performance of $AgNO_3$ to callus induction and regeneration was well studied for some graminaceous species (Adkins et al. 1993, Fei et al. 2000). Among all additives and their concentrations, satisfactory results on callus induction (53.33%) and regeneration (55.66%) were observed when 10 mg/l of $AgNO_3$ were added to the optimum callus-inducing- and regeneration medium. However, better result was observed in callus induction (73%) and plantlet regeneration (61.33%), when all the four additives and growth regulators were added to the medium. Our results on the effect of additives either individually or in combination are in agreement with those reported earlier by Gray and Conger (1985), Trigiano and Conger (1987), Prakash et al. (1999), Lee et al. (2003) showing that each additive has some specific function on callus induction and shoot regeneration.

Carbon source is one of the key factors in *in vitro* tissue culture system. Fuentes et al. (2000) reported that specificity of carbohydrates may have differential effects on morphogenesis. Even though it is well documented that sucrose is the best source of carbon for *in vitro* system, there are also some reports where maltose was found to give better results than other carbohydrates (Ainsley and Aryan 1998, Chang et al. 2003, Sharma et al. 2005). We, therefore,

investigated the role of different sources of carbon on callus induction and regeneration. Significant variation was observed among the carbohydrates tested. Sucrose induced more callusing, i.e., 73%. Maltose produced 7% more regenerants, raising the percentage from 61 to 68%. In *in vitro* system maltose breaks down slowly and provides a metabolizable carbon source over a longer period than sucrose (Orshinsky et al. 1990). When present in the medium it acts primarily as a nutritional source rather than an osmoticum (Fuentes et al. 2000). Superiority of maltose promoting callusing and regeneration via somatic embryogenesis has been widely reported (Ainsley and Aryan 1998; Chang et al. 2003; Sharma et al. 2005).

It is reported that the basal medium plays a significant role in callus induction and somatic embryogenesis (Ainsley and Aryan 1998, Zhao et al. 1999, Lee et al. 2002). In the present investigation MS, N_6 and SH were used under optimum combinations of growth regulators, additives and carbon sources. Significant variations on callus induction and plant regeneration were observed among the basal media. About 73% seeds callused on MS, whereas the highest percentage (71.66) of shoot regeneration was observed on N_6. Performance of SH on callus induction and plantlet regeneration was much lower than MS and N_6. Variations of supplements such as nitrogen source or other chemical combinations or their varying concentrations added to different basal media as supplements are attributed as possible causes for differential tissue culture responses (Ainsley and Aryan 1998).

Genotypic variations in *in vitro* responses are frequently observed in grass species (Akula et al. 1999, Bai and Qu 2000, Chaudhury and Qu 2000, Fei et al. 2000, Lee et al. 2003, Zale et al. 2004, Sharma et al. 2005). Similar to earlier reports, our results also revealed that callus induction and plantlet regeneration frequency are dependent on genotypic variation among the nine cultivars.

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