식물생명공학회지 제34권 제2호
J Plant Biotechnol
Vol. 34, No. 2, 161-166 (2007)

In Vitro Plant Regeneration from Stolon Node Explant in *Eremochloa Ophiuroides* (Munro) Hack

Shyamkumar Barampuram, Byung Yeoup Chung*, Seung Sik Lee, Byung Chull An, and Jae-Sung Kim

Advanced Radiation Technology Institute (ARTI), Korea Atomic Energy Research Institute (KAERI), 1266 Shinjeong-dong, Jeongeup 580-185, Korea

ABSTRACT *In vitro* shoot regeneration and multiple shoot induction has been obtained from the stolon node explants in *Eremochloa ophiuroides* (Munro) Hack. The highest number of shoots (10.66 ± 0.21) was observed from initial explants after one month culture duration on Murashige and Skoog (MS) medium containing 6-benzyladenine (BA: 0.5 mg/l). First generation shoot was excised and sub-cultured on the same fresh media for further multiplication of shoots. An enhanced number of second round shoots (15.33 ± 0.21) was obtained compared to the initial culture media containing BA (0.5 mg/l). The number of shoots/stolon node was higher among all the concentrations of BA than kinetin (KN). *In vitro* regenerated shoots were successfully rooted in the phytohormone free MS medium. Plantlets generated with roots were transferred to pots containing compound mixture of soil and kept in green house conditions. Acclimatized plants showed 100% survival rate with normal morphology in green house conditions. The present study demonstrates the effect of explant and different plant growth regulators towards *in vitro* response in *E. ophiuroides.* Moreover, the study reveals the effect of cytokinin on induction of shoot number per stolon node explant in *E. ophiuroides.*

Introduction

Eremochloa ophiuroides (Munro) Hack, is commonly known as centipedegrass, and belongs to the family Poaceae. Centipedegrass is a warm season creeping perennial and one of the most popular turf grass around the world (Islam and Hirata 2005a). Centipedegrass is widely distributed in South-east Asia, USA, South America, Europe, West Indies, Africa and North and East part of Australia (Hook et al. 1992; Duble 1996). Centipedegrass is light green in color, with leafy stolons and compressed sheath, which lie flat against or on the soil surface and resemble centipede (Islam and Hirata 2005b, Bao and Hirata 2006). The stolons of centipedegrass are slender and branching with internodes of 10-35 mm and holds roots on them. The grass forms a relatively short and highly dense sword with rapidly spreading stolons (Islam et al. 2004a, b). It has been reported that centipedegrass is dormant during winter and rejuvens growth slowly in spring and expands rapidly in summer (Cai et al. 2004).

Centipedegrass is more feasible to use in lawns, parks and golf course turfs. It has been reported that, this grass is convenient to use on the road sides, industrial and other low maintenance areas (McCarty 1995, Landry and Murphy 2002, Islam and Hirata 2005a). Due to its rapidly growing leafy stolon and high dense forming sward, this grass is convenient to use in soil conservation, particularly in high rainfall and sloping areas (Islam and Hirata 2005a). In recent times in Japan, this grass is used as ground cover in rice fields to inhibit weeds (Fuke et al. 2006). Besides the

*Corresponding author Tel 063-570-3331 Fax 063-570-3339
E-mail: bychung@kaeri.re.kr

above uses, the leaf of this grass contains important compounds such as chlorogenic acid (CA), luteolin and maysin (Wiseman et al. 1990). Among these compounds, maysin is very effective against fall army worm larvae. Maysin is an unusual luteolin glycoside that possesses a unique keto-sugar (Gueldner et al. 1991). It has been demonstrated that CA and maysin are the major factors responsible for the antibiotic resistance of centipedegrass to larvae of the fall armyworm (Johnson et al. 2002).

Being one of the popular turfgrass with variety of uses, it lacks a suitable *in vitro* direct plant regeneration protocol. There was only one report available on plant regeneration via callus phase in centipedegrass (Krans and Blanche 1985). However, to our knowledge there are no reports on direct plant regeneration from stolon node explant in centipedegrass. Development of *in vitro* plant regeneration protocols is a pre-requisite for genetic transformation studies. It has been suggested by Wang and Ge (2006), that stolon node transformation is highly applicable and more advantageous than callus phase. Moreover, the generation of transformants from the same genotype obtained through stolon node transformation excludes possible genotypic effects in the regenerants. In the recent times, transformation procedures using stolon nodes were developed in bermudagrass, creeping bentgrass and zoysiagrass (Wang and Ge 2005, Ge et al. 2006). To best of our knowledge, there is no report available on direct plant regeneration from stolon node and also on information regarding the effect of various plant growth regulators as culture media supplements in centipedegrass tissue culture. Therefore, in the present study, we have attempted to evaluate the *in vitro* response of explant to different cytokinins and direct regeneration from the stolon node explants in centipedegrass.

Materials and Methods

Plant Material

The plant materials such as stolon nodes were collected from centipedegrass plants growing in green house condition at Advanced Radiation Technology Institute (ARTI), KAERI, Korea.

Multiple Shoot Induction and Plant Regeneration

Plant sample was processed by removing excess leaves and cut into size of 3-4 cm with node and subjected to the sterilization process. Explants were washed with 0.5% (v/v) soap solution (LG Household & Health Care, Korea) for 5 min and kept under tap water for 1h. Surface sterilization of explants was carried out in laminar flow with 0.1% (w/v) carbendazim (Sigma-Aldrich, USA) for 10 min, followed by 6-8 washes with sterile distilled water. Explants were further surface sterilized with 0.1% (w/v) mercuric chloride (Sigma-Aldrich, USA) for 2 min and rinsed with sterile distilled water for 6-8 times.

Explants were trimmed at both ends with sterile scalpel and placed horizontally on hormone free MS (Murashige and Skoog 1962) media containing 2% (w/v) phytagel and 3% (w/v) sucrose and media supplemented with various concentrations of 6-benzyladenine (BA: 0.5, 1.0, 2.0 and 4.0 mg/l), Kinetin (KN: 0.5, 1.0, 2.0 and 4.0 mg/l) and combination of both BA + KN (0.5 + 0.5, 1.0 + 1.0 mg/l). The pH of the media was adjusted to 5.8. All the media were autoclaved at 1.06 $kgcm^{-2}$ pressure for 15 min. All the cultures were incubated in growth room at temperature of 24 ± 2°C, 16 h photoperiod and irradiance 70 $\mu mol\ m^{-2}s^{-1}$.

A single first generation shoot was excised and subcultured in the same respective media combinations for second round of shoots. *In vitro* regenerated shoots of length 7-8 cm were excised and grown in hormone free MS media for rooting. Shoots with well developed roots were washed with tap water to free from agar and transferred to pots containing soil mixture (peat moss + vermiculite + perlite 1:1:1). Acclimatization of rooted plants was carried out in green house conditions at 24 ± 2°C, relative humidity 90%.

Statistical Analysis

Six explants were used for each treatment, and each treatment was repeated for a minimum three times with similar culture parameters. Observation such as the number of shoots per explant was recorded after one month of culture. The data were subjected to one way ANOVA and LSD multiple comparison test was performed using SPSS statistical package

(version 11.0). Values are expressed as mean ± S.E. *P* value < 0.01 was considered significant.

Results

Shoot Regeneration, Multiplication and Rooting

A gradual increase and elongation of the shoot from the stolon node was observed in explants after 2-3 days of inoculation invariably among all the combinations evaluated (Figure 1A). Further, after 8-10 days of culture, initiation of new shoots was observed adjacent to the initial shoot from nodal end on media supplemented with various concentrations of BA or KN (Table 1, Figure 1B). However, it took a minimum of 15-16 days for the formation of new shoots from explants inoculated on hormone free MS media. A varied response in terms of shoot regeneration from stolon node explant was observed among various concentrations of cytokinins tested (Table 1). The highest number of shoots (10.66 ± 0.21) was obtained on media supplemented with 0.5 mg/l BA (Table 1, Figure 1C, D). A number of shoots formed were found significant among various concentrations of BA, when compared to other media combinations. Media containing 0.5 mg/l KN showed the highest number of shoots (4.66 ± 0.21) from initial culture when compared to other concentrations of KN. However, the shoots formed were not much significant among various concentrations of KN evaluated (Table 1).

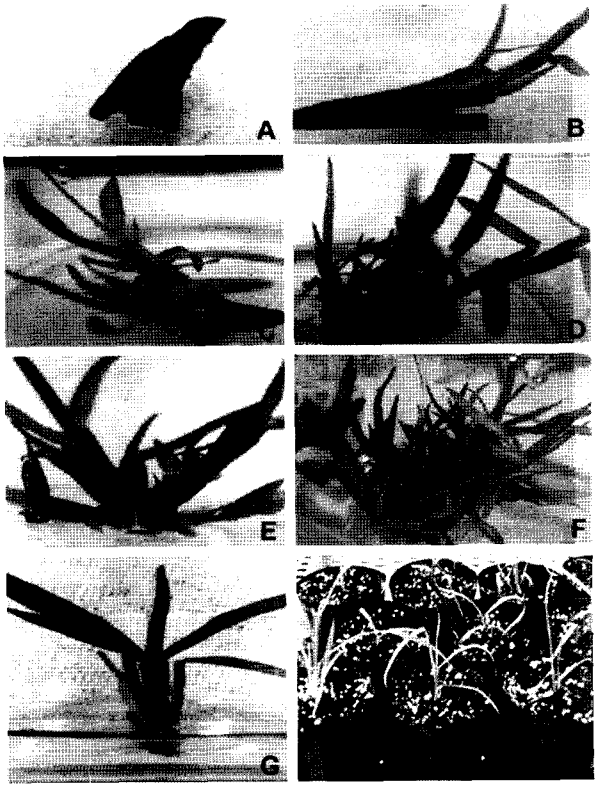

Figure 1. Plant regeneration and multiple shoot induction from stolon node explants of *Eremochloa ophiuroides*. (A) Stolon node explant of size 3-4 cm, cultured on MS + BA (0.5 mg/l) for multiple shoot induction; (B) Elongation of the stolon explant with new shoots after 10 days of culture response; (C) Further growth of the shoots after 2 weeks of culture response; (D) Multiple shoots induction after 4 weeks of culture from stolon node explant; (E) Induction of new shoots adjacent to the *in vitro* grown first generation shoot after 2 weeks of culture; (F) The highest number of second round multiple shoots (15.33 ± 0.21) was obtained after 4 weeks of culture on MS + BA (0.5 mg/l); (G) Rooting of the shoot in hormone free MS media; (H) Acclimatized plants growing in green house conditions.

Among two different cytokinins, BA was found to be better than KN, with respective to the various concentrations tested (Table 1). There was a decline in the shoot number per explant with increase in hormonal concentration of both cytokinins i.e. BA and KN. There was a varied response observed in terms of length of the shoots among the different hormonal combinations tested (data not shown). The length of the shoots formed was higher on hormone free MS media compared to the media with hormones. Media containing

Table 1. Effect of different cytokinins on shoot induction from stolon node explants in *Eremochloa ophiurodes*

Media	Plant growth regulators		No. of shoots / explant after one month of culture Mean ± S.E
	BA (mg/l)	KN (mg/l)	
MS	-	-	4.83 ± 0.16
MS	0.5	-	10.66 ± 0.21***
MS	1.0	-	7.83 ± 0.40***
MS	2.0	-	7.33 ± 0.21***
MS	4.0	-	6.33 ± 0.51***
MS	-	0.5	4.66 ± 0.21^{ns}
MS	-	1.0	4.50 ± 0.22^{ns}
MS	-	2.0	4.16 ± 0.16^{ns}
MS	-	4.0	3.33 ± 0.21***
MS	0.5	0.5	4.22 ± 0.16^{ns}
MS	1.0	1.0	2.83 ± 0.21***

MS: Murashige and Skoog, BA: 6-benzyladenine, KN: Kinetin Values expressed are Mean ± S.E for six replicates. *** P < 0.01 was considered significant. ns- Not significant as compared with MS.

combination of two hormones i.e. BA and KN didn't show any improvement in terms of shoot number per explant (Table 1). Moreover, the length of the shoots formed was less and stunted (data not shown).

First generation shoots were excised and inoculated in the same respective media combinations for second round of shoots. A new shoot initiation was observed adjacent to the inoculated shoot within 6-8 days of culture, irrespective of various media combinations tested. However, the number of shoots formed was varied among different media combination evaluated (Table 2). The highest number of second generation shoots i.e., (15.33 ± 0.21) was observed on media containing BA (0.5 mg/l) (Table 2, Figure 1E, F). The number of shoots formed was comparatively higher than first generation shoots on media containing BA (0.5 mg/l) (Table 2). Among various concentrations tested with BA, the number of shoots formed was found comparatively higher than KN. However, there was an increase in shoot number among all the concentrations of KN compared to its initial culture (Table 2).

The initiation of roots from shoot was observed within 10 days on hormone free MS media. Morphology of the roots was long, slender with lateral roots (Figure 1G). Alternatively, rooting experiment was carried out with different auxins such as indole-3-acetic acid (IAA), indole-3-butyric acid (IBA). However, there was a varied response observed among various hormone concentrations in terms of the average number of roots per shoot and percentage of rooting (data not shown). After one month of culture, the shoots with well developed roots were washed with tap water to remove bounded agar around the roots and transferred to pots containing soil. The survival percentage of the plantlets as recorded after 4 weeks of transfer to soil was 100% (Figure 1H).

Table 2. Effect of different cytokinins on second generation of shoots from *in vitro* grown shoot of *Eremochloa ophiurodes*

Media	Plant growth regulators		No. of shoots / explant after one month of culture Mean ± S.E
	BA (mg/l)	KN (mg/l)	
MS	-	-	4.66 ± 0.33
MS	0.5	-	15.33 ± 0.21 ***
MS	1.0	-	9.66 ± 0.21 ***
MS	2.0	-	9.50 ± 0.22 ***
MS	4.0	-	7.83 ± 0.30 ***
MS	-	0.5	6.56 ± 0.21***
MS	-	1.0	6.33 ± 0.22 ***
MS	-	2.0	6.16 ± 0.34 ***
MS	-	4.0	4.16 ± 0.21^{ns}
MS	0.5	0.5	7.16 ± 0.16***
MS	1.0	1.0	5.22 ± 0.40^{ns}

MS: Murashige and Skoog, BA: 6-benzyladenine, KN: Kinetin Values expressed are Mean ± S.E for six replicates. *** $P < 0.01$ was considered significant. ns- Not significant as compared with MS.

Discussion

In the present study, it was clear that lower concentration of cytokinins (BA and KN) were found favourable in terms of higher multiple shoot induction per explant. The results also revealed that inclusion of low level BA showed higher number of shoots (10.66 ± 0.21 and 15.33 ± 0.21) than media with higher concentration. In one of the report, lower concentration of BA was found to be significant in plant regeneration in augustine grass (Li et al. 2006). It has also been reported that BA was found effective for direct plant regeneration from stem explants in Kentucky bluegrass (Hu et al. 2006; Colomba et al. 2006). In contrast, KN was found better than BA in terms of initiation of shoot from stolon node in zoysiagrass (Ge et al. 2006). Although the stolon node inoculated on hormone free MS medium resulted in shoot formation, the initiation of shoot took longer duration when compared to the media with hormone. The number of shoots formed was also very low when compared to the media containing 0.5 mg/l BA (Table 1 and 2). This clearly indicates the supplement of cytokinin not only enhanced shoot number, but also resulted in faster shoot initiation from stolon node on hormone containing media than MS basal medium. During establishment of cultures, root initiation was observed from the nodal part of the stolon among all the combinations evaluated. This is one of the morphological characters of the centipedegrass observed under in vivo conditions (Islam and Hirata 2005a).

In the present study, the shoots formed from the explants

represent the response capacity of the explant to the hormone concentrations and the number of shoots per explant represents the capacity of the explants to produce shoots. The supplement of exogenous plant growth regulators probably depends on the specific endogenous hormone levels (Bhaskaran and Smith 1990). Media supplemented with two cytokinins were found significant in second round of multiple shoots than the initial culture. Although there was increase in the shoot number compared to its initial culture the length of shoots formed were short, stunted and thin. Decline in the shoot number with increase in the hormonal concentration reveals that there was a hormonal imbalance between the exogenous and endogenous levels and inhibit the growth of shoots per explant. Overall, BA was found to be the best cytokinin in terms of high shoot induction from initial and first generation shoot explant. The rooted plants were healthy, green and 100% rooting was observed among all the replicates. Similarly, in one of the report the regenerated plants were rooted in hormone free media in bermudagrass (Jain et al. 2005). All the plants were found healthy, green and showed normal morphology characteristics in green house conditions.

In conclusion, as there is less literature about in vitro and no literature about direct regeneration in centipedegrass, the present work may provide valuable information to develop an efficient plant regeneration protocol in centipedegrass. The *in vitro* regenerants obtained in the present protocol may serve as a good source for protoplast for genetic improvement in centipedegrass. Application of stolon node regeneration system for direct transformation reduces possible genotypic effects in the regenerants than obtained through callus phase. Moreover, the stolon node transformation system will be more advantageous, effective and much applicable to centipedegrass.

Acknowledgements

This project has been carried out under the Nuclear R&D Program of the Ministry of Science and Technology, Korea.

References

Bao GZ, Hirata M (2006) Effects of defoliation frequency on the development and establishment of a vegetatively planted turfgrass *Eremochloa ophiuroides* (centipedegrass). Trop Grasslands 40: 102-110

Bhaskaran S, Smith RH (1990) Regeneration in cereal tissue culture: a review. Crop Sci 30: 1328-1336

Cai Q, Wang S, Cui Z, Sun J, Ishil Y (2004) Changes in freezing tolerance and its relationship with the contents of carbohydrates and proline in over wintering centipedegrass (*Eremochloa ophiuroides* (Munro) Hack.). Plant Prod Sci 7: 421-426

Colomba EL, Grunberg K, Griffa S, Ribotta A, Mroginski L, Biderbost E (2006) The effect of genotype and culture medium on somatic embryogenesis and plant regeneration from mature embryos of fourteen apomictic cultivars of buffel grass (*Cenchrus ciliaris* L.). Grass Forage Sci 61: 2-8

Duble RL (1996) Turfgrasses-Their management and use in the Southern Zone, 2nd ed., Texas A & M University Press, College station, Texas, pp. 1-336

Fuke K, Hozumi N, Enami Y, Matsuura K, Tajimi Y (2006) Anthracnose of centipedegrass caused by *Colletotrichum caudatum*. J Gen Plant Pathol 72: 74-75

Ge Y, Norton T, Wang ZY (2006) Transgenic zoysiagrass (*Zoysia japonica*) plants obtained by *Agrobacterium* -mediated transformation. Plant Cell Rep 25: 792-798

Gueldner RC, Snook ME, Wiseman BR, Widstrom NW, Himmelsbach DS, Costello CE (1991) Maysin in corn, teosinte and centipede grass. In: Hedin PA (ed), Naturally occurring pest bioregulators, ACS Symposium Series No. 449. Am Chem in the various turfgrasses Soc, Washington DC, pp. 251

Hook JE, Hanna W, Maw BW (1992) Quality and growth response of centipedegrass to extended drought. Agron J 84: 606-612

Hu XR, Yang AF, Zhang KW, Wang J, Zhang JR (2006) Optimization of *in vitro* multiple shoot clump induction and plantlet regeneration of Kentucky bluegrass (*Poa pratensis*). Plant Cell Tiss Org Cult 84: 89-98

Islam AM, Hirata M (2005a) Centipedegrass (*Eremochloa ophiuroides* (Munro) Hack.): Growth behaviour and multipurpose usages. Grassl Sci 51: 183-190

Islam MA, Hirata M (2005b) Leaf appearance, death and detachment, and tillering in centipedegrass (*Eremochloa ophiuroides* (Munro) Hack.) in comparison with bahiagrass (*Paspalum notatum* Flugge): A study at a small sod scale. Grassl Sci 51: 121-127

Islam MA, Hirata M, Bao G (2004a) Leaf dynamics of centipedegrass (*Eremochloa ophiuroides* (Munro) Hack.) in comparison with bahiagrass (*Paspalum notatum* Flügge):

a preliminary study at a small sod scale. Grassl Sci 50 (Suppl.): 88-89

Islam MA, Hirata M, Bao G (2004b) Sward structure and leaf dynamics of centipedegrass (*Eremochloa ophiuroides* (Munro) Hack.): a study at a pasture scale. Grassl Sci 50 (Suppl.): 90-91

Jain M, Chengalrayan K, Gallo-Meagher M, Mislevy P (2005) Embryogenic callus induction and regeneration in a pentaploid hybrid bermudagrass CV. Tifton 85. Crop Sci 45: 1069-1072

Johnson AW, Snook M.E, Wiseman BR (2002) Green leaf chemistry of various turfgrasses: Differentiation and resistance to fall armyworm. Crop Sci 42: 2004-2010

Krans JV, Blanche FCB (1985) Tissue culture of centipedegrass. In: Lemaire F (ed) Proc. Int. Turfgrass Res. Conf., 5th, Avignon, France, 1-5 July 1985. Int. Turfgrass Soc and INRA, Paris, pp. 159-164

Landry G, Murphy T (2002) Centipede Lawns. Leaflet 313, Cooperative Extension Service. The University of Georgia College of Agricultural and Environmental Sciences, Georgia, pp. 1-4

Li R, Bruneau AH, Qu R (2006) Improved plant regeneration and *in vitro* somatic embryogenesis of St. Augustinegrass (*Stenotephrum secundatum* (Walt) Kuntze). Plant Breeding 125: 52-56

McCarty LB (1995) Centipedegrass for Florida Lawns. Fact Sheet ENH-8(a), Florida Cooperative Extension Service. Institute of Food and Agriculture Science, University Florida, Florida, pp. 1-2

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plantarum 15: 473-497

Wang Z-Y, Ge Y (2006) Recent advances in genetic transformation of forage and turf grasses. In Vitro Cell Dev Biol-Pl 42: 1-18

Wang Z-Y, Ge Y (2005) Rapid and efficient production of transgenic Bermuda grass and creeping bent grass bypassing the callus formation phase. Funct Plant Biol 32: 769-776

Wiseman BR, Gueldner RC, Lynch RE, Severson R.F (1990) Biochemical activity of centipedegrass against fall armyworm larvae. J Chem Ecol 16: 2677-2690

(Received June 8, 2007; Accepted July 15, 2007)