African Journal of Biotechnology Vol. 11(1), pp. 138-144, 3 January, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2521
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Reproducible and expedient rice regeneration system using *in vitro* grown plants

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Accepted 15 December, 2011

Inevitable prerequisite for expedient regeneration in rice is the selection of totipotent explant and developing an apposite combination of growth hormones. Here, we reported a reproducible regeneration protocol in which basal segments of the stem of the *in vitro* grown rice plants were used as ex-plant. Using the protocol, callus was developed from the exposed cells of root segments and cortical tissues of basal part of the stem. Various levels of 2,4-dichlorophenoxyacetic acid (2,4-D) were used, where 1 mg/L was found as best level for callus induction. Further, 25 combinations of kinetin and naphthalene acetic acid (NAA) were developed to investigate the regeneration response of the calli. The root-derived calli did not respond to any combination at all, whereas the nodular calli derived from the stem segments responded variably to kinetin and NAA combinations in the Murashige and Skoog (MS) medium from base to top. Higher kinetin to NAA ratios promoted embryogenesis, whereas lower ratios exhibited rhizogenesis. A combination of 3 mg/L kinetin and 1 mg/L NAA was established to be the best combination for plant regeneration through embryogenesis in rice from *in vitro* grown plants. Plants regenerated *in vitro* were successfully acclimatized in the pots, where they exhibited phenotypically indistinguishable normal growth when compared with plants developed from seed, hence the developed regeneration system for rice in these studies may be treated as one of the best strategies to *in vitro* clonal propagation and purification, ahead of seasonal growth of plants in the field or green house.

Key words: Rice, *in vitro* regeneration, explants, naphthalene acetic acid (NAA), kinetin, rhizogenesis.

INTRODUCTION

Rice is the major food crop in Asia and the rest of the world, and thus, varieties with improved characteristics are desired. Rice, with its relatively small genome size (389 Mb, International Rice Genome Sequencing Project, 2005), well developed genetics, availability of a dense physical map and molecular markers together with its complete genome sequence is considered a model monocot system for genetic engineering and functional genomic studies (Bajaj and Mohanty, 2005). Using rice as a model, monocot system is critical for food safety, hunger eradication and poverty alleviation (Coffman et al., 2004). With the development of plant molecular biology and genetic engineering, its transformation has become one of the core issues.

Inevitable prerequisite for expedient regeneration in rice is the selection of totipotent explant (Hoque and Mansfield, 2004) and developing an apposite combination of growth hormones. Efforts have been made to use various explants, such as protoplast derived cell suspension culture (Tang et al., 2001), mature seeds (Khanna and Raina, 1997; Saharan et al., 2004; Bano et al., 2005), the radicals (Mikami and Kinoshita, 1988), the coleoptiles, mature embryos (Khanna and Raina, 1998),

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Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; **NAA,** naphthalene acetic acid; **PGR,** plant growth regulators; **KOH,** potassium hydroxide; **HCl,** hydrochloric acid; **CRD,** completely randomized design.

root segments (Hoque and Mansfield, 2004) and microspores (Genovesi and Magill, 1982) for callus induction and subsequent rice regeneration. To date, only suspension culture of the proliferating embryogenic calli (Khan and Maliga, 1999) and mature seed scutellum derived calli (Lee et al., 2006) have been used in plastid transformation studies of rice. The significance of the relative levels of two major categories of plant growth regulators (PGR), the cytokinins and auxins in plant development under *in vitro* conditions, has long been comprehended. Exogenous application or determination of endogenous levels of plant growth regulators concluded that regeneration of various patterns of differentiation, that is, embryogenesis, organogenesis or rhizogenesis from *in vitro* cultured tissues can be induced by varying the cytokinin-to-auxin ratio in the growth medium (Smigocki and Owens, 1989). Here, we report a reproducible regeneration protocol in which basal segments of the stem of the *in vitro* grown rice plants were used as explant. Present research was carried out by keeping in view the importance of *in vitro* plants as explant source and demonstrates an effective regeneration method for the purification of engineered lines as well as for prompt multiplication of plants with reduced period of propagation using simplified media, aiming to improve the regeneration of stable transformed cells at levels of nucleus as well as plastids (Khan and Maliga, 1999; Khan et al., 2001; Bock, 2001; Maliga, 2004; Daniell et al., 2002).

MATERIALS AND METHODS

Plant

Mature seeds of rice cultivar Super Basmati (*Oryza sativa* L. ssp *indica*) were obtained from the Rice Research Institute, Kala Shah Kaku, Lahore, Pakistan. Seeds were dehusked manually in order to avoid detachment of embryos from seeds. Seeds were surface sterilized with 70% (v/v) ethanol for 30 s and rinsed thrice with deionised water. Further surface sterilization was carried out on a shaker using 50% (v/v) commercial bleach for 30 min and rinsed four to five times with sterilized water in a laminar air flow chamber (Priya et al., 2011). The seeds were cultured on wet filter paper under light for germination. Five days old germinated seedlings were shifted to sterile magenta boxes containing MSO (MS basal medium supplemented with sucrose 30 g/L) for further growth to establish plants of suitable stem girth.

Callus induction

Callus induction from basal stem segments

In vitro grown plants were sacrificed after 30 to 40 days. Their roots were removed and the lower most part of stem (Figure 1 A) was sectioned into thin discs of about 1 to 1.5 mm thickness. Each of the basal stem portions was sliced into almost six to eight discs (cross sections). These discs were cultured on the callus induction media and were incubated in the dark at 25 ± 2°C. The explants were cultured onto a modified MS (Murashige and Skoog, 1962) medium containing MS macro and micro salts, thiamine HCl 0.10 mg/L, pyridoxine HCl 0.50 mg/L, nicotinic acid 0.50 mg/L, myo-inositol 100 mg/L, glycine 2 mg/L and sucrose 30 mg/L for callus induction (Khan and Maliga, 1999) supplemented with five concentrations of 2, 4 dichlorophenoxyacetic acid (2,4-D) as 1, 2, 3, 4 and 5 mg/L. The media were solidified with phytagel (2.6 g/L) and the pH was adjusted to 5.8 with KOH/HCl prior to autoclaving. All the chemicals were purchased from *Phyto*Technology Laboratories® USA, except phytagel which was purchased from Sigma USA.

Callus induction from root

Roots of *in vitro* grown plants (Figure 1A) were also excised with a sharp scalpel into 0.25 to 0.5 cm length. These root segments were also cultured onto same callus induction medium as mentioned earlier (for stems explants) and were incubated in dark at 25 ± 2°C.

Regeneration

Three weeks old dark induced calli derived from *in vitro* grown stem discs and root segments were cultured onto regeneration media and were incubated in 16 h photoperiod (fluorescent light tubes providing irradiance of 170 µmol $m^{-2}s^{-1}$) and temperature of 25 ± 2°C. 25 different concentrations and combinations of plants growth regulators (kinetin and naphthalene acetic acid (NAA)) were tested in order to explore maximum regeneration potential of these calli (Table 1). Total number of shoots/explant was counted after five weeks of culture. When regenerated plantlets had well-developed root system, they were transferred to pots filled with peat moss and soil for acclimatization.

Microscopy

Callus induction, regeneration initiation and somatic embryogenesis were observed under a stereo microscope (SZX-10; Olympus Co., Ltd.) and were photographed by a digital camera (DP-20; Olympus Co., Ltd). All images were arranged with Adobe Photoshop CS software (Adobe Systems Inc).

Experimental design and statistical analysis

A completely randomized design (CRD) with three replications per treatment was used in this study. Each replication contained three plates of respective media. In one plate, all the basal stem segments of one seedling were arranged and numbered sequentially so that the effect of position of basal stem segments of each seedling may be compared. Mean value of three plates was considered as one replication. In callus induction and subsequent regeneration experiment, due to predominance of zeros, an ANOVA was not applicable; hence, we were confined to the calculation of means and standard errors of means (SEM) only (Table 1).

RESULTS

Seed germination and seedling growth

Sterilized seeds after germination were inoculated on MSO for germination. Seed started to germinate after three days of inoculation. Seven days old, healthy seedlings were transferred to the jars on the same medium for further growth.

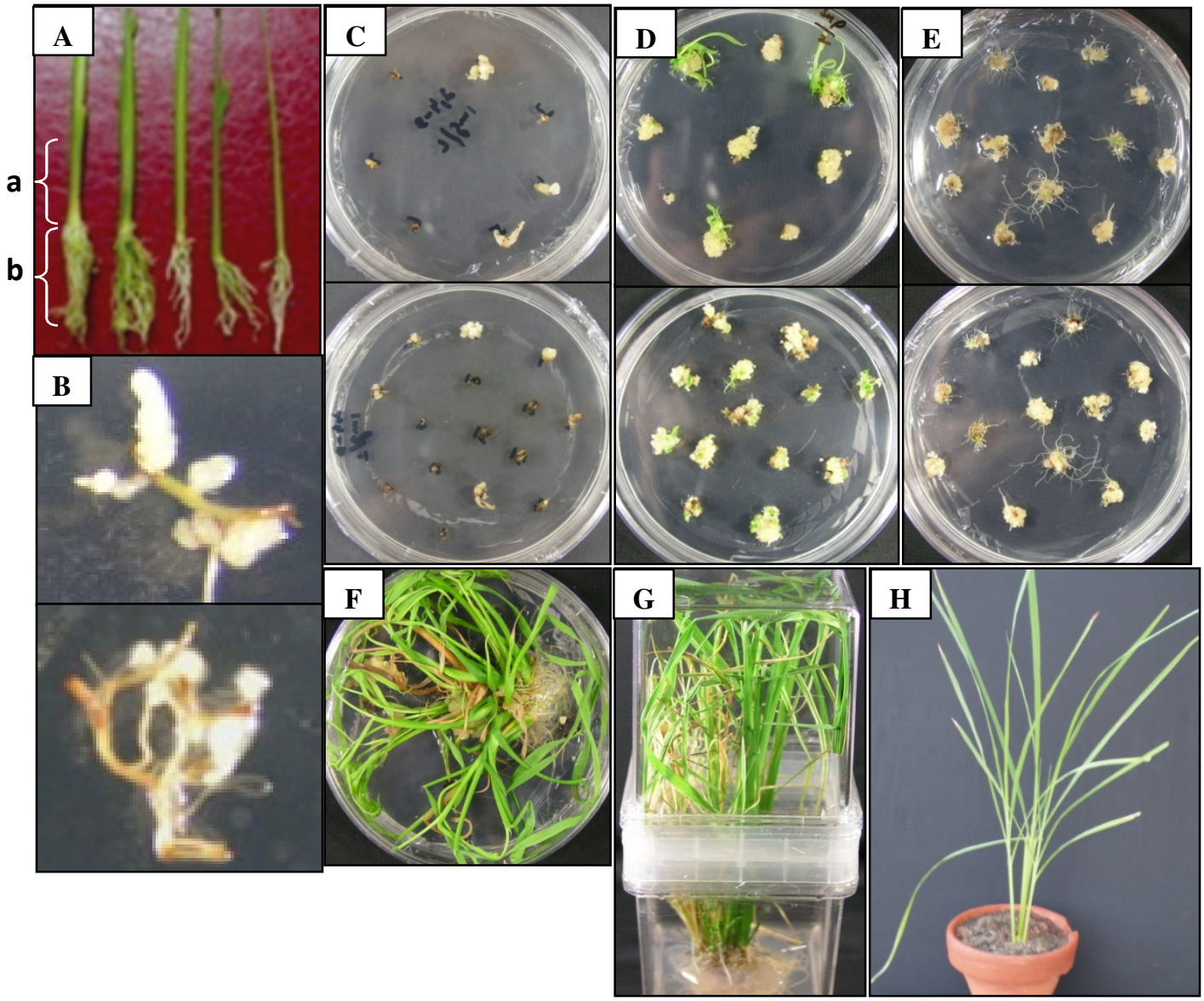

Figure 1. Callus induction and subsequent regeneration of rice using *in vitro* grown explants. A, Source of explants (a) stem segments and (b) root segments used as explants; B, callus induction visible on cut ends of root segments; C, basal stem discs cultured sequentially on callus induction medium; D, embryogenesis and subsequent green plants regenerated from basal stem disc derived calli; E, rhizogenic response of basal stem disc derived calli; F-G, *in vitro* plant proliferation for fully expanded leaves and well developed roots; H, acclimatization of *in vitro* grown rice plants.

Table 1. Regeneration response of *in vitro* grown rice explant.

NAA (mg dm^{-3})	Kinetin (mg dm^{-3})				
	1	2	3	4	5
0.1	3.66 ± 1.2	2.66 ± 1.2	1.33 ± 0.9	0.00 ± 0.0	0.00 ± 0.0
0.5	3.66 ± 0.3	2.66 ± 0.9	3.00 ± 1.2	3.66 ± 0.3	2.00 ± 0.6
1	0.00 ± 0.0	5.33 ± 0.9	9.66 ± 2.0	5.66 ± 0.8	3.00 ± 0.9
2	0.00 ± 0.0	0.00 ± 0.0	2.66 ± 0.8	5.33 ± 0.3	3.33 ± 0.2
3	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	3.66 ± 0.3	2.66 ± 0.3

The shoots emerging from each stem segments on various kinetin and NAA concentrations were counted. Data represents the average values of three independent experiments of three replicate each and at least seven callus segments cultured per replicate. NAA, Naphthalene acetic acid

Callus induction

Callus derived from root segments

Calli initiated from root segments cultured on callus induction media supplemented with 1 to 5 mg/L of 2,4-D (Figure 1B). Calli started to emerge five days after inoculation. Callus induction was observed only from the cut ends of primary root segments and on the tips of secondary roots by virtue of root apical meristems present on their apices. The calli increased in size and sometimes covered the whole segment of secondary root. No part of primary root segments developed calli except the cut ends. Furthermore, calli produced on root segments were detached and transferred to the regeneration media with a variety of PGR combinations (Table 1). No callus segment regenerated into green plants, rather, creamy white color of calli turned brown leading to the necrosis of the whole callus mass.

Callus derived from basal stem portion

Sections of the basal part of the stem of 1 to 1.5 mm thickness of *in vitro* grown rice plants were cultured onto callus induction media supplemented with 1 to 5 mg/L of 2,4-D. Calli were initiated from 2nd lower most section (Figure 1C) of 1 to 1.5 mm thickness. All the other stem sections (below and above) never responded to callus induction. It was a general observation that callus induction frequency decreased by increasing the 2,4-D concentration in callus induction media. It was maximum at 1 mg/L, while minimum at 5 mg/L of 2,4-D. Thickness of stem sections also appeared to have significant impact on callus induction as callus induction rate decreased with increase/decrease in thickness than the optimized 1 to 1.5 mm. Light microscopy of calli also revealed that callus was induced from the cortical region of stem having parenchymatous and collenchymatous tissues (Figure 2A). The calli became visible 5 to 7 days after inoculation.

Regeneration response

Embryogenic response of calli developed from basal stem sections

Calli that developed from the basal stem sections of *in vitro* grown plants were transferred to the regeneration media augmented with 25 combinations of kinetin and NAA in order to explore its regenerability. A varied response to different combinations was observed where calli either continued proliferating on one medium, while another medium favored embryogenesis or rhizogenesis. Among these combinations, kinetin (3 mg/L) and NAA (1 mg/L) appeared to be the most promising as far as regeneration is concerned with a maximum (9.67 ± 2.02) shoots per explant (Table 1). Embryogenic calli became green in color within two to three weeks of culture and produced green plantlets (Figure 1D). It was also observed that few combinations favored rhizogenesis (Figure 1E) instead of embryogenesis. Light microscopy of calli derived from the cortical region of basal stem discs (Figure 2A), regenerating callus exhibited greening of cells (Figure 2B) and somatic embryo formation (Figures 2C and D). Green plants with well developed roots (Figures 1F and G) were shifted to pots having a mixture of peat moss and soil, for acclimatization and subsequent transfer to the field. Almost 90% of the plants successfully acclimatized (Figure 1H). Figure 3 depicts the overall trend of the effects of kinetin to NAA ratios on the regeneration of rice plants from basal stem disc derived calli.

Rhizogenic response of calli developed from basal stem sections

Auxin to cytokinin ratios are known to have significant effect on the patterns of differentiation (Smigocki and Owens, 1989). Calli proliferated from the basal stem sections of *in vitro* grown plants were transferred to the regeneration medium supplemented with kinetin and NAA in various ratios (Table 1). High NAA to kinetin ratios favored root induction instead of shoot formation. Hence, under these conditions, calli never developed shoots while profused rooting was observed (Figure 1E).

DISCUSSION

In these studies, a reproducible regeneration system was developed using mature green explants where basal parts of the *in vitro* grown plants were used to develop shoots on MS medium supplemented with auxin and cytokinin in varied ratios (Skoog and Miller, 1957). Under *in vitro* conditions, environmental stimuli must be integrated with intrinsic developmental signals by virtue of a group of structurally unrelated small molecules, the plant growth regulators. It is very obvious that plant growth regulators interact with one another at multiple levels during plant growth and development (Santner et al., 2009) by affecting each other's synthesis, transport and/or response. The type of interaction may be synergistic, antagonistic or additive (Coenen and Lomax, 1997) depending upon the tissue, its developmental stage and culture conditions (Jaillais and Chory, 2010). Together, these mechanisms coherently coordinate developmental decisions (Busch and Benfey, 2010) culminating into callogenesis, organogenesis or embryogenesis. Investigations into cross-talk among plant growth regulators were underpinned by early discoveries on the antagonistic effects of auxins and cytokinins (Skoog and Miller,

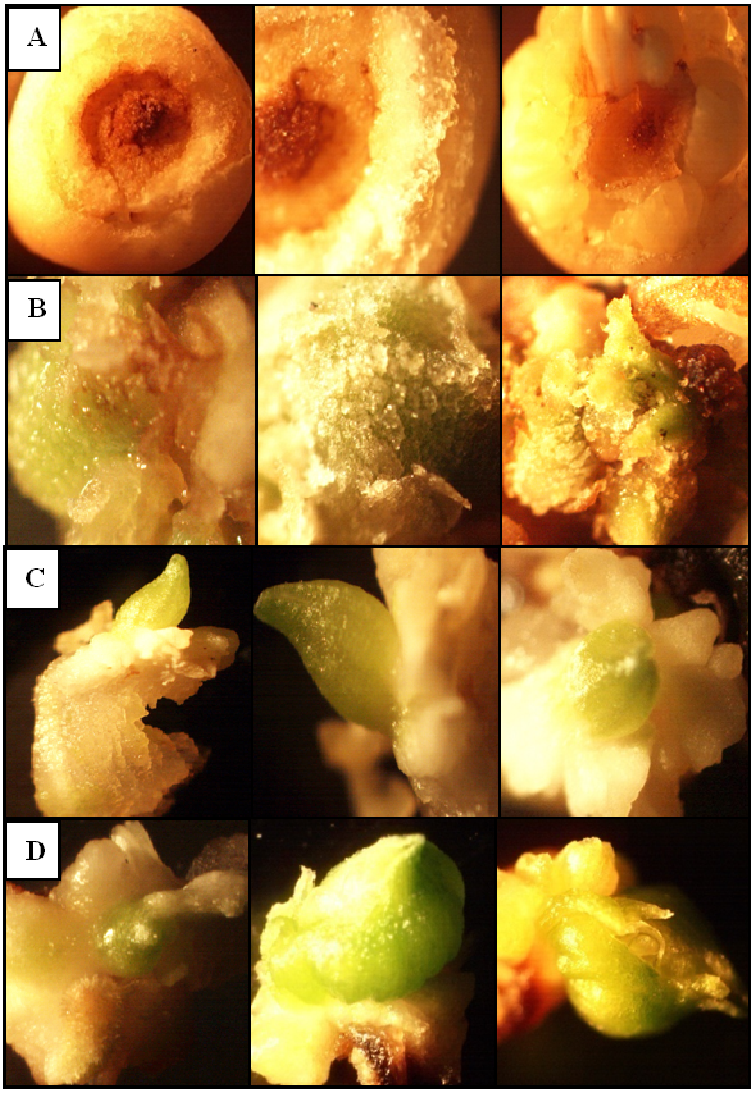

Figure 2. Light microscopy of callus induction and subsequent regeneration of calli induced from basal stem discs. A, Calli derived from the cortical region of basal stem discs; B, basal stem discs derived calli on regeneration medium showing greening of embryogenic calli; C-D, somatic embryos derived from the basal stem discs derived calli.

1957). The present study has also demonstrated that the auxin to cytokinin ratio is decisive to *in vitro* response of plant tissues. Exposing callus cultures to a high ratios of auxin:cytokinin resulted in root formation, conversely (Skoog and Miller, 1957) whereas, a low ratio promoted somatic embryogenesis as auxins are known to regulate cytokinin pool size and vice versa (Nordstrom et al., 2004; Taele et al., 2008). Auxins and cytokinins interact at the level of hormone perception and signal transduction in plants in order to control many core developmental processes (Müller and Sheen, 2008; Moubayidin et al., 2009; Jones et al., 2010). Cytokinins themselves are signaling molecules that exert a widespread influence over plant growth and development (Mok, 1994). Usually, cytokinins are negative regulators of root growth and development (Werner et al., 2003). Auxins are also thought to exert control over the cell cycle by regulating key genes (Blilou et al., 2002; del Pozo et al., 2002). The cell cycle is therefore under the influence of both hormones. Hence, auxin:cytokinin ratio is important for the control of many developmental processes, including organ regeneration from differentiated tissue. The studies described here are in accordance with the classical studies of Skoog and Miller (1957). Outer stem portion, the cortex, of young *in vitro* grown plants being green in color contains the chloroplasts in chlorenchyma tissues. The cortical chlorenchyma is structurally (Bossard and Rejmanek, 1992; Yiotis et al., 2006) and functionally similar to the palisade and/or spongy mesophyll in leaves; hence, active in

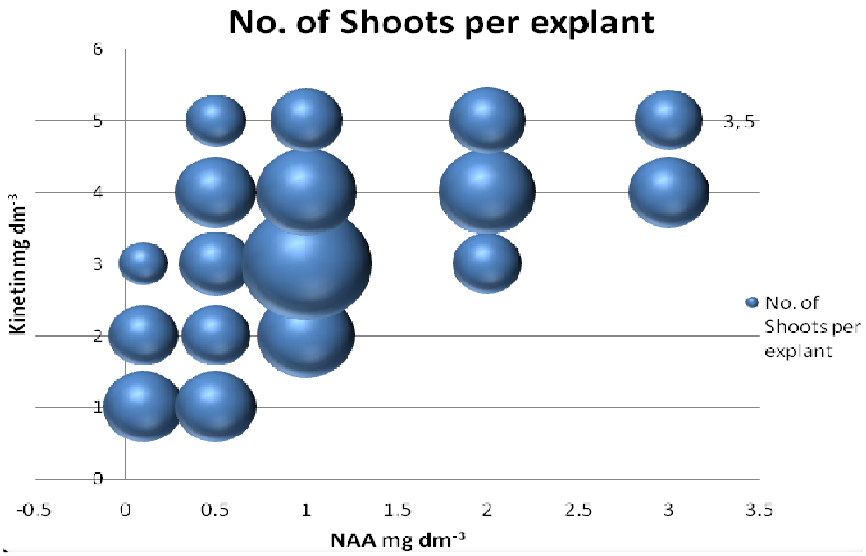

Figure 3. Regeneration of rice plants from basal stem disc derived calli when cultured on regeneration media. Area occupied by the balls show the number of shoots per explant on respective kinetin and NAA concentrations. All data represent the average values of three independent experiments.

photosynthesis (Nilsen, 1995) and contain mature chloroplasts (Yiotis et al., 2006). Therefore, green stem portions if made amenable to *in vitro* manipulations will become preferred explant for plastid transformation and/or clonal purification, because it requires 16 to 17 continuous cycles of selection and regeneration (Khan and Maliga, 1999; Moll et al., 1990) using currently available seed based tissue culture protocols. We describe here a protocol that promoted callogenesis followed by regeneration from mature cells of green stem. This kind of tissue culture system will be of realistic importance in plastid transformation protocols in future. Efforts have been made for callus induction and subsequent regeneration from green explants in many cereals like maize (Ahmadabadi et al., 2007), Oat (Chen et al., 1995; Gless et al., 1998), wheat (Haliloglu, 2006), sugarcane (Franklin et al., 2006) and rice (Ramesh et al., 2009). According to the best of our knowledge this is the first report on regeneration system where green explant from base of the stem portion of was used to regenerate fertile plants of indica rice cultivar. Furthermore, the established protocol offers tightened frame period of *in vitro* regeneration system as it avails explant after one month when compared with conventional seed based rege-neration system that requires 5 to 6 months to get into next generation of the same plant.

Cereal crops being the world's most important food source are the preferred target of genetic manipulation for crop improvement. This has raised environmental concerns, most of which are related to transgene outcrossing and their uncontrolled spreading via pollen to related weeds resulting obnoxious contamination of the food chain. Therefore, the development of a plastid transformation system for cereal crops is highly enviable owing to maternal inheritance of plastids (Bock and Khan, 2004; Maliga, 2004; Daniell et al., 2005). Furthermore, for the purification of transplastomic lines 16 to 17, continuous cycles of selection and regeneration are required to attain homoplasmicity, the ultimate goal of any plastid transformation event. There is no hurdle to perform these many cycles of selection and regeneration in dicots to purify genome since leaf-based regeneration systems, for example in tobacco, are available. Contrarily, no such systems are available to regenerate plants from leaves in rice. Previous studies (Lee et al., 2006) have shown that it was impossible to convert the transplastomic rice plants to homoplasmy, even after two generations of continuous selection using mature seed based regeneration system. Therefore, it necessitates devising a strategy to purify the transformed plastid genome of rice. Hence, a reproducible regeneration protocol is developed in which basal segments of the stem of the *in vitro* grown rice plants were used as explant. The protocol will be helpful in developing stable transgenic plastids through rapid purification of clones with improved characteristics.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Ministry of Science and Technology and Higher Education Commission (HEC), Pakistan for providing funds and Rice Research Institute, Kala Shah Kaku Lahore, Pakistan for seed.

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