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Promotive factors for callus initiation and plant regeneration in upland rice

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ABSTRACT

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Factors promotive to the initiation of embryogenic callus and regeneration of plants in upland rice were investigated using mature dehulled seeds. The objectives are to enhance the production of embryogenic callus and increase regeneration of plants. In upland rice, light incubation as well as 30 g/L sorbitol and 50 mg/L tryptophan were promotive for the formation of embryogenic callus and green shoot buds when added in the rice callus initiation (RCI) medium during the initial culture stage. Likewise, the combinations of 0.5 mg/L NAA and 0.5 mg/L BAP or, 0.5 mg/L IAA and 0.5 mg/L BAP supplement in the rice plant regeneration (RPR) medium enhanced the production of green shoot buds and plants from callus that were precultured in RCI medium containing 1.0 mg/L abscisic acid. The upland rice regenerants exhibited phenotypic variation from their parental counterparts in the field.

Keywords: callus initiation, culture medium, plant regeneration, promotive factors, upland rice

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INTRODUCTION

Rice is an important component of food security for upland farmers. Nearly 100 million people now depend on upland rice as their staple food. In the Philippines, 2% of the approximately 3.4 million hectares of rice lands is upland. Yet genetic improvement of upland rice lag way behind the lowland rice[1].

Shoot and plant regeneration has been achieved from different somatic tissues in rice (reviewed by Sree Rangasamy *et al.* 1992). However, most of these were on lowland rice. The published procedures are seldom applicable to upland rices due to their highly variable culture response. Regardless of the explant source, all of the approaches have focused nearly on the establishment of a regeneration system via callus induction. This has proven to be a multi-step procedure which requires a long time interval for the development of whole plants (Raina and Irfan, 1998; Stricklen, 1991; Abe and Futsuhara, 1986). So far, there is no report that dealt with improvement of culture conditions that could be applied to a wide range of rice varieties and genotypes. Hence, search for new promotive factors such as organic additives, amino acids, plant hormones as well as culture conditions must be done to improve embryogenic callus formation and plant regeneration in upland rice. The ability to regenerate plants from isolated cells or tissue underpins most selection and plant transformation systems.

In this study, the effects of promotive factors including light and dark culture condition, culture medium additives such as sorbitol, tryptophan, glutamine, abscisic acid and plant growth regulators on the initiation of embryogenic callus and regeneration of plants were investigated using mature dehulled seeds. The objective is to enhance the production of embryogenic callus and, increase the production of green shoot buds and regeneration of whole plants.

MATERIALS AND METHODS

Preparation of Seed Explants

Mature seeds from seven upland rice varieties namely; Azucena,

Dinorado, Lubang Red, Magkaling, PSB Rc1, UPL R5, and UPL R7 were obtained from the Philippine Rice Research Institute (PhilRice), Munoz, Nueva Ecija, Philippines. The seeds were manually dehulled and soaked at 100 seeds/ 50 ml of 70% (v/v) ethyl alcohol for 30 sec after which the ethyl alcohol was decanted. The seeds were decontaminated by soaking in 50% of commercial bleach solution (Zonrox) for 15 min. During decontamination, the seeds were agitated every 5 min after which the bleach solution was decanted. The seeds were rinsed with six changes of autoclaved distilled water prior to inoculation on the culture medium.

Induction of Callus

The decontaminated dehulled seeds were sown at five seeds per 20 ml of the rice callus initiation (RCI) medium placed in 50 ml Erlenmeyer flask. The RCI medium was composed of Murashige and Skoog's (1962) salts, Gamborg's (1968) B5 vitamins, 30.0 g/L sucrose, 1.0 mg/L 2,4-dicholorophenoxy acetic acid (2,4-D) and 0.5 mg/L kinetin, and solidified with 6.0 g/L agar-agar (Pronadisa, Hispan Lab., Manila) at pH 5.8. The medium was autoclaved at 15 psi at 121°C for 20 min then, cooled prior to use.

Two factors were investigated during the callus induction experiment namely; (1) the effect of light and dark incubation condition and, (2) the effect of sorbitol supplement in the RCI medium.

1. Effect of Light and Dark Incubation

The effect of continuos light or darkness on callus induction of seeds was determined. The first batch of decontaminated seeds sown at five seeds per flask was incubated on open culture shelves provided with continuous light coming from a 40-W cool white fluorescent tube. The second batch of seeds was incubated on culture shelves covered with black cloth. The calli that were later on induced from the scuttelar node of the newly-germinated rice seedlings were plucked off and transferred to fresh RCI medium for 2-3 weeks to induce further proliferation. These calli were categorized into two

types: the embryogenic type (E) and the non-embryogenic type (NE).

2. Effect of Sorbitol

Sorbitol at 30 g/L was added into the RCI medium at three different culture stages; (1) during the initial culture of seeds up to the second passage (up to 9 weeks after seed inoculation), (2) at callus proliferation stage (or at 4th week of incubation) up to second passage, and (3) at callus maintenance stage (or at 7th week of incubation) until the formation of 'green spots' or green shoot bud primordia from callus (at 10th week of incubation). All seed cultures were maintained inside a culture room provided with 25 ± 2 °C temperature for about three weeks to induce callusing.

Regeneration of Plants

The effects of culture medium additives; α-glutamine and α-tryptophan, abscisic acid (ABA) and plant growth regulators such as indole acetic acid (IAA), naphthalene acetic acid (NAA), benzylaminopurine (BAP) and their combinations were investigated to improve plant regeneration.

1. Effect of α -glutamine and α -tryptophan

α -glutamine at 800 mg/L and α-tryptophan at 50 mg/L were added separately or combined into the RCI medium at the first passage (4 weeks after inoculation) and second passage (7 weeks after inoculation) to increase the formation of E callus. Cultures were incubated under continuous diffuse daylight (1.0 Wm2) provided by 40-W cool white fluorescent tube at 25 ± 2 °C for 3 weeks. The effects of single addition of α-glutamine or tryptophan or, combined α-tryptophan and α-glutamine on E calli formation and plant regeneration were determined.

2. Effect of Abscisic Acid

ABA at 1.0 mg/L was added into the RCI medium containing 30 g/L sorbitol at three different culture periods namely; (1) at first passage (4th week of incubation), (2) at second passage (7th week of incubation), and (3) at third passage (10th week of incubation). Calli from first passage and second passage were incubated in the dark at 25 ± 2 °C for the initiation and proliferation of callus. Calli from the third passage were incubated under 16h photoperiod of 4.0 Wm^2 provided by daylight fluorescent tubes for the regeneration of plants.

3. Effect of Auxin and Cytokinin Supplement

E calli were subcultured into 30 mL aliquots of agar-solidified regeneration (RPR) medium composed of half strength MS salts, B5 vitamins, 30 g/L sucrose and 30 g/L sorbitol at pH 5.8. The RPR medium was supplemented with combinations of either IAA (0.2 and 0.5 mg/L) and BAP (0.5 and 1.0 mg/L), or NAA and BAP using the same range of concentrations. The calli were incubated under 16h of daylight provided by 40-W cool white fluorescent tube at 25 ± 2 °C, for the production of green plants.

Rooting, Potting and Field Planting

The regenerated upland rice shoots were transferred for 2-3 weeks in agar-solidified halfstrength MS medium containing 30 g/L sucrose to improve rooting. Then, the well-rooted plantlets were acclimatized (while still inside the culture bottles) for 1-2 weeks at room temperature under a 24-h light provided by a 40-W cool white fluorescent tube. After the acclimatization period, the plantlets were taken out of the culture bottles then, the agar medium was removed by washing the roots with tap water. The roots were dipped in a weak fungicide solution (0.1 % solution of Benlate) and then, the plantlets were planted in clay pots filled with soil. The newly-transplanted plantlets were covered with transparent plastic cups to maintain high relative humidity

and prevent the plantlets from drying up. The potted plantlets were watered whenever necessary. One hundred upland rice regenerants were transplanted in a rainfed area at one plant per hill to observe agronomic characteristics.

RESULTS AND DISCUSSION

Induction of Callus

1. Effect of Light and Dark Incubation

Rice seeds cultured in RCI medium with 1.0 mg/L 2,4-D and 0.5 mg/L kinetin developed a coleoptile from one day onward (Fig. 1a). The scutellum region of the embryo began to swell and then the whole embryo increased in size followed by the onset of callus formation (Fig. 1b). Callus was initiated from the scutellar surface of the seed after 5-7 days of inoculation. The callus clump was a mixture of E and NE callus. The E callus was interspersed with NE callus, though both were morphologically distinguishable. E callus was firm with irregular and nodular surface, opaque, light yellow, contained a highly dense cytoplasm and potentially regenerable (Fig. 1c). The NE callus was friable, white or translucent to brownish, with large vacuolated cells and non-regenerable. When further subcultured, the periphery of NE calli turned brown and became necrotic (Fig. 1d). The variable appearance and regeneration potential of E and NE calli implies that there are differences in gene expression between the two callus types (Chen and Luthe, 1987).

The upland rice varieties; Azucena, Dinorado, Lubang Red, Magkaling, PSB Rc1, UPLR5 and UPLR7 exhibited genotypic variability in RCI medium similar to that reported by Abe and Futsuhara (1986). Since the incubation condition is an important factor affecting the induction of E callus (Nabors and Dykes 1985), two sets of seed cultures were maintained separately under light and dark condition. After 4 weeks of incubation, 52.5-97.5% of the seed cultures initiated callus under light while, 40.0-61.3% formed callus in the dark (Fig. 2a). The light condition seemed to favor callus formation from the seeds. Among the upland rice varieties, 'Dinorado' and Lubang Red' varieties showed the highest percentage of callus formation.

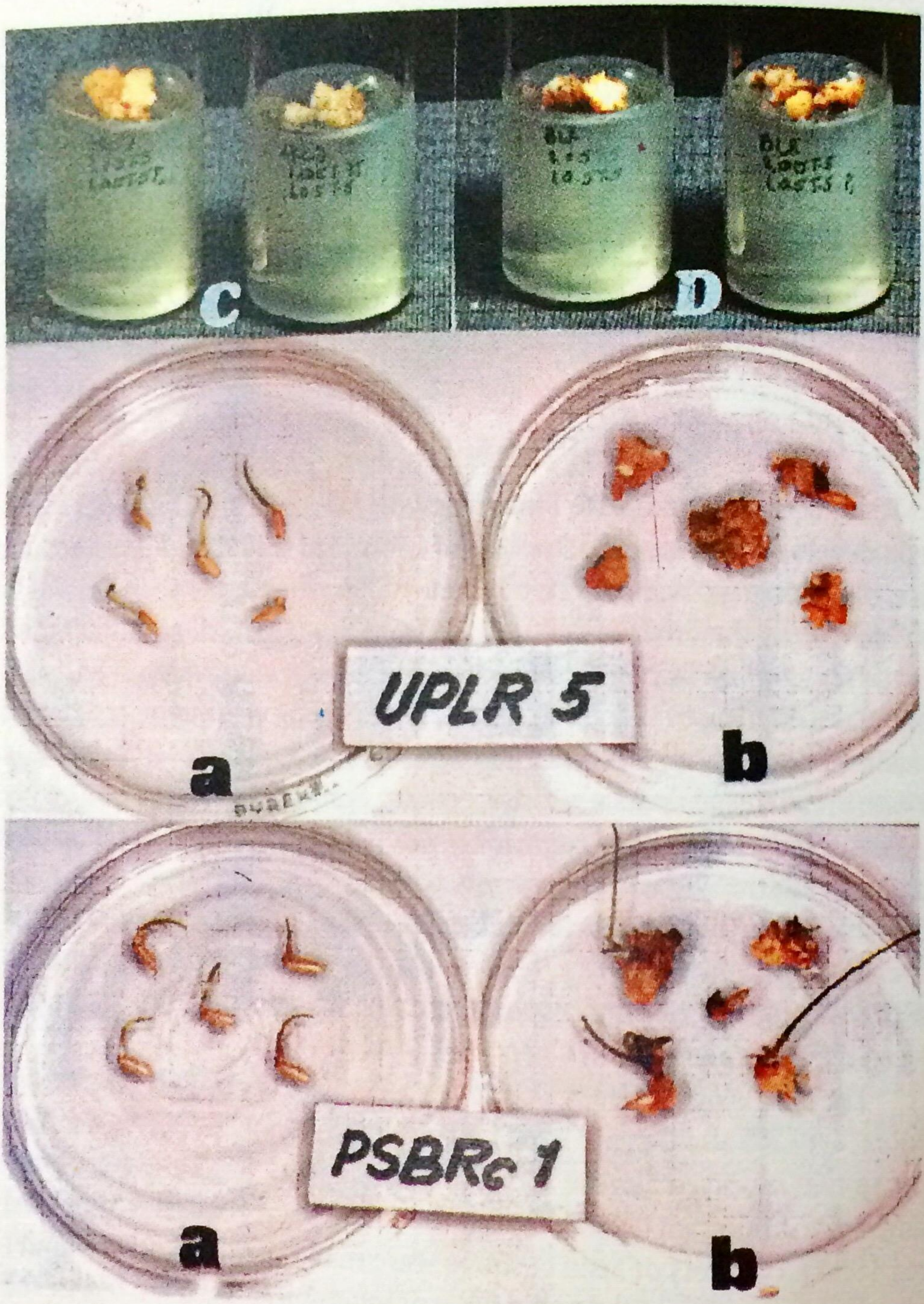

Fig. 1. Initiation of callus from mature dehulled seeds of upland rice. (a) emergence of coleoptile, (b) callusing of the scutellar region, (c) embryonic callus, and (d) non-embryonic callus.

There was no significant difference between light and dark incubation in the formation of E callus except in the variety 'Magkaling'. The 'Magkaling' variety showed the highest percentage of seeds forming E callus under light (Fig. 2b). Both E and NE calli had the ability to proliferate in the medium containing 1.0 mg/L 2,4-D and 0.5 kinetin. After 3 weeks of incubation, E calli were subcultured into fresh medium while the NE calli were discarded.

2. Effect of Sorbitol

PSB Rc1, UPLR 5, and UPLR7 varieties were used in investigating the effect of sorbitol supplement in the culture medium because in the previous experiment, these varieties exhibited lower percentage of E callus formation compared to other varieties. In this experiment, incorporation of 30 g/L sorbitol in the RCI medium substantially increased the percentage of seeds forming callus under light incubation. Sorbitol generally exerted profound effect when added during callus initiation stage than during callus proliferation or maintenance stage (Fig. 3a). This is shown by high percentage of E callus formation (Fig. 3b) and 'green spot' production (Fig. 3c). Figure 4a shows the formation of E calli from UPLR 7 seeds which were interspersed with NE calli. The E calli were separated from NE calli and, the E calli were subcultured on fresh fresh RCI medium containing 30.0 g/L sorbitol. After 3 weeks of incubation, shoot buds which appeared as 'green spots' were initiated from the E calli (Fig. 4b). The 'green spots' developed into complete plants after 3 weeks of incubation in RPR medium (Fig. 4c). This result shows that sorbitol can penetrate into the rice callus to act as osmotic agents acting against the creation of a critical turgor pressure which must be established before cell expansion can occur (Kavi Kishor and Reddy 1986). The enhancing effect of sorbitol however, was less when it was added during the callus proliferation and maintenance stage. It is therefore suggested to incorporate 30 g/L sorbitol starting from the callus initiation stage until the regeneration stage. Sorbitol supplement also maintained the morphogenetic competence of E callus for two passages resulting to green spot production. This however, does not always guarantee high frequency plant regeneration.

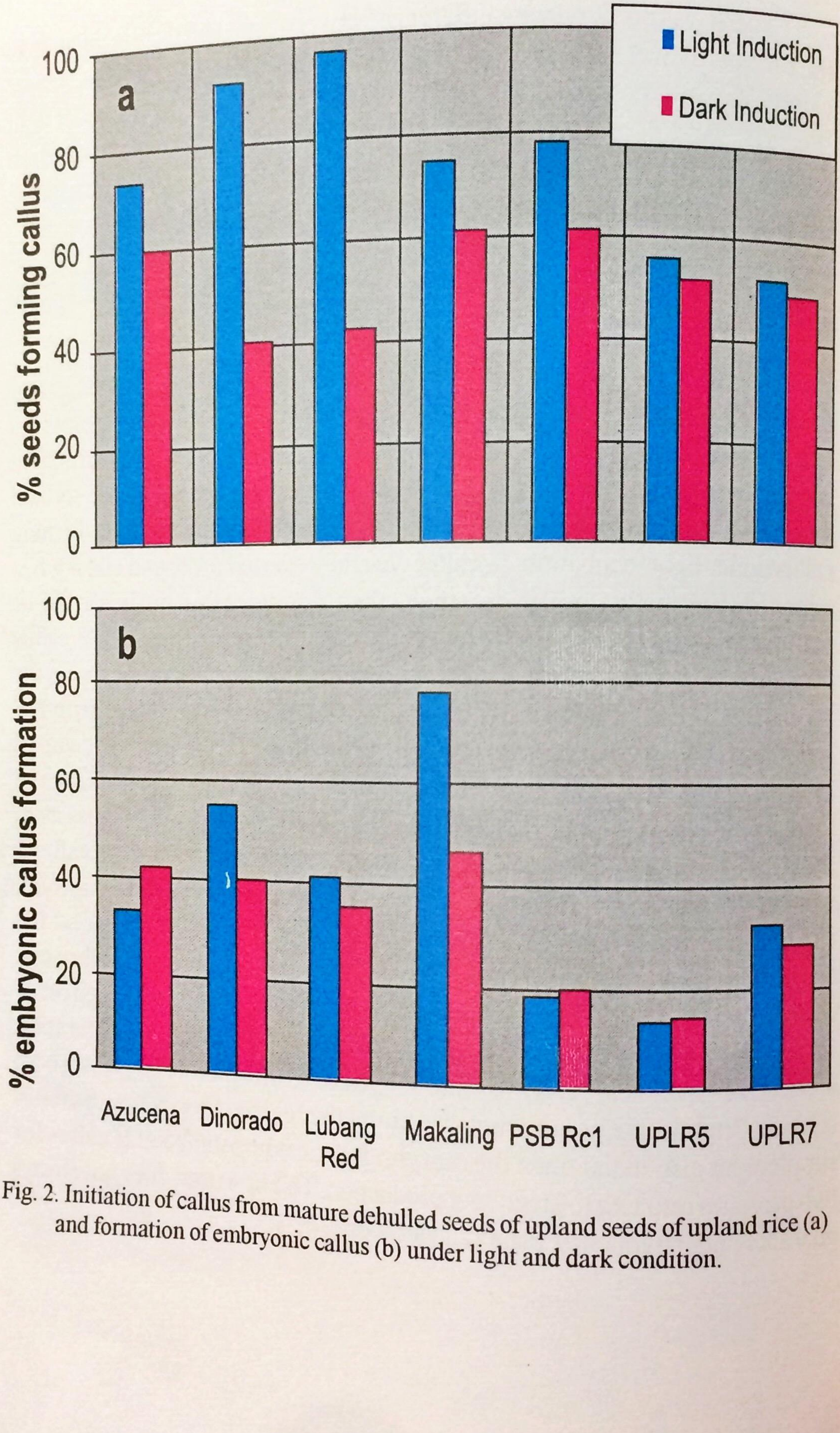

Fig. 2. Initiation of callus from mature dehulled seeds of upland seeds of upland rice (a) and formation of embryonic callus (b) under light and dark condition.

Regeneration of Plants

1. Effect of α-glutamine and α-tryptophan

Increasing plant regeneration frequency is the ultimate aim of many tissue culture procedures. Various organic additives, plant hormones as well as amino acids (Chowdhry *et al.* 1993; Mathew and Philip, 2003) have been reported to enhance regeneration of plants from cells, tissue and organs. In this experiment, the addition of 50 mg/L tryptophan into the RCI medium increased the percentage of callus formation from seeds of PSB Rc1, UPLR5 and UPLR7 varieties compared to glutamine or, glutamine and tryptophan supplement (Fig. 5a). This conforms with the findings of Ling and Yoshida (1987) who reported that the addition of 50mg/L or 100 mg/L tryptophan induced a high frequency of green-plant regeneration in rice. Regardless of the upland rice variety used in this study, tryptophan increased the percentage of E callus formation (30-45%) compared to glutamine (10-20%) or, glutamine and tryptophan combination (15-22%) (Fig. 5b). Consequently, a higher percentage of green spot production (30-35%) was observed from cultures supplemented with tryptophan (Fig. 5c). The promotive effect of tryptophan in somatic embryogenesis and plant regeneration of rice was also reported by Chowdhry *et al.* (1993) and Sree Rangansamy *et al.* (1992). In this experiment, low callusing and poor green shoot bud production in RCI medium supplemented with glutamine contradicts the reports of Redway *et al.* (1990) in rice and Belarmino *et al.* (1994) in sweet potato protoplasts.

The sequential stages from the initiation of E callus to the formation of green spots and regeneration of complete plants in PSB Rc1 and UPLR5 are shown in Figure 6. In PSB Rc1 variety, shoots and roots were produced simultaneously (Fig. 6a) whereas, in UPLR5 variety shoot initiation occurred prior to rooting (Fig. 6b). There was 10-60% plant regeneration from upland rice varieties PSB Rc1, UPLR5 and UPLR7 cultured in RPR medium containing 0.5 mg/L IAA and 0.5 mg/L BAP and 50 mg/L tryptophan. Peterson and Smith (1991) also observed increased regeneration using tryptophan but only in combination with BAP in the subculture medium. In this experiment, the

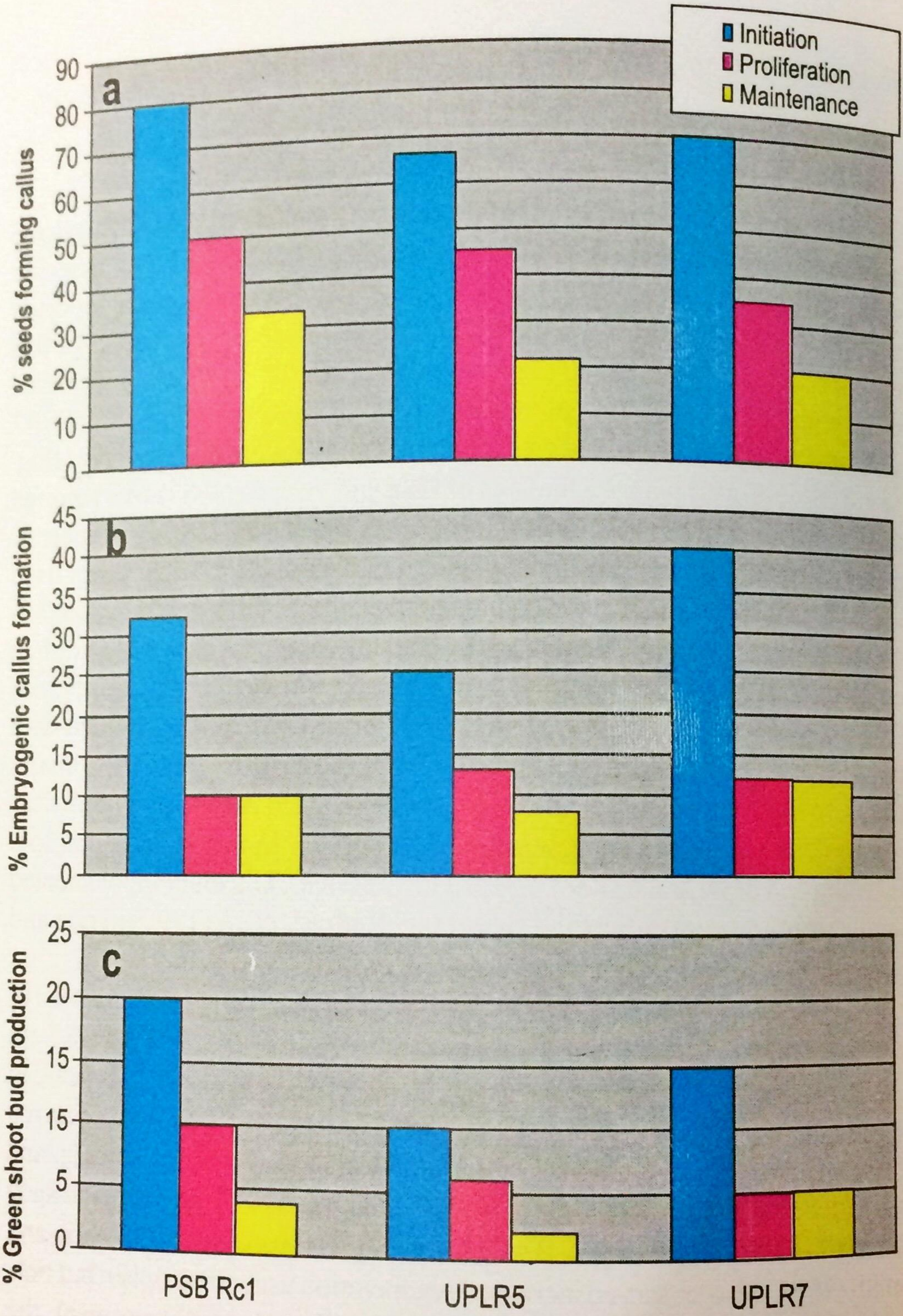

Fig. 3. Initiation of callus from upland rice seeds (a), embryogenic callus formation (b), and green shoot bud production (c) as influenced by the addition of 30/L sorbitol at different culture stages.

Fig.. 4.Callus induction and plant regeneration in upland rice variety UPLR7. (a) formation of embryogenic and non-embryogenic callus mixture, (b) production of green shoot bud primordia, (c) regeneration of green pants.

combination of IAA and BAP plus tryptophan favored regeneration of plants. The fact that tryptophan can serve as precursor of IAA has led to the proposal that tryptophan acts by altering endogenous levels of the hormone, IAA (Siriwardana and Nabors 1983).

2. *Effect of Abscisic Acid*

Stimulation of shoot bud and plant formation by ABA was demonstrated in rice (Torrizo and Zapata, 1986) and other species (Shepard 1980; Belarmino *et al*., 1997; Saunders and Tsai, 1999). In this study, ABA added at the second passage or at the 7th week of culture in the RCI medium induced higher percentage of E callus formation compared when ABA was added at the initiation stage or first passage (Fig. 7a). The E calli produced at the second

Table 1. Comparative performance of upland rice regenerans and seed-derived parentals in the field

Upland rice variety	Ave. plant height (cm)	Ave. no. of tillers/plant	Ave. no. of panicle/plant	No. of harvested panicle/plant	Ave. weight of panicle (g)
Regenerants[1]					
PSB Rc1	99.5	22.0	18.9	13.9	34.4
UPLR5	117.5	11.5	9.3	8.8	27.5
UPLR7	106.4	9.4	8.0	9.0	30.0
Parentals			6.0	5.2	18.0
PSB Rc1	113.1	11.5			
UPLR5	153.2	12.3	11.5	6.0	24.7
UPLR7	122.5	8.2	9.0	5.0	22.0

[1] One hundred plants were transplanted for each variety

passage were compact, nodular, opaque, yellow and embryogenic compared to the small and translucent or pale white calli produced during the initiation and first passage. These calli developed into plantlets after 3 weeks of culture in RPR medium. It is evident that incorporation of low concentration of ABA in the preculture medium (prior to transfer to the regeneration medium) maintained morphogenetic competence of E calli (Ling and Yoshida 1987; Sree Ramaswamy *et al.*, 1992)) thereby, sustaining the development of 'green spots' or shoot buds into complete plants (Li and Wolyn, 1995; Saunders and Tsai, 1999). In this experiment, 15-30% green shoot bud production was observed from callus cultures precultured in ABA- containing RCI medium (Fig. 7b). The regeneration of plant was not observed during the ABA treatment but after transfer of E calli to the ABA-free regeneration (RPR) medium. The initiation of green shoot bud primordia in E calli was the first indication of callus differentiation and regenerative activity. This observation was also mentioned by Ben Amer and Borner (1997) who suggested a significant positive correlation between green spot initiation and plant regeneration in cereals. Thus, selecting only calluses producing early green spots will increase regeneration frequency.

3. *Effect of Auxin and Cytokinin Supplement*

Various combinations of IAA and BAP or, NAA and BAP were added into the RPR medium to determine the effective levels for plant regeneration. Raghava Ram and Nabors (1983) reported that medium containing low levels of BAP, sometimes low levels of IAA, and lacking 2,4-D promotes regeneration of rice plants. In this experiment, PSB Rc1, UPLR 5 and UPLR 7 varieties showed varied responses in the RPR medium supplemented with different concentration of IAA and BAP or, NAA and BAP combinations inspite of good callusing performance (Fig. 8a). Among the different concentrations that were tested, 0.5 mg/L IAA and 0.5 mg/L BAP and, 0.5 mg/L NAA and 0.5 mg/L BAP induced regeneration of plants from callus (Fig. 8b) however, the number of plants regenerated from each variety differed (Fig. 8b). The variation in regeneration efficiency may be attributed to differences in the components and concentrations of endogenous phytohormones present in a variety, and differences in the susceptibility of such variety to auxin and cytokinin ratio. Almost all plants survived after potting out (Fig. 8c).

A direct connection of root and shoot axis (photo not taken) was indicative of regeneration via somatic embryogenesis, which is the preferred mode of differentiation (Maheswari *et al.* 1990). Unlike those reported by Raina *et al.* (1987) and Oard and Rutger (1988), there were no albinos among the regenerants in this experiment. Rooting of the regenerants was improved by transferring them to agar-solidified half strength MS medium containing 30 g/L sucrose (Fig. 9a). Almost all plants survived the 4-week hardening in the greenhouse (Fig. 9b) and were successfully transplanted in the field (Fig. 9c).

The regenerants were generally shorter than the parentals (Table 1). Among the varieties used, only PSB Rc1 showed high tillering and panicle-bearing abilities and higher panicle weight that was approximately twice that of the parentals (Table 1). The regenerants flowered approximately at the same time as the parentals. Callus-derived rice regenerants exhibited phenotypic variation similar to those reported by Sree Rangasamy et al. (1992).

Considering the above-mentioned findings, two culture media; rice callus initiation (RCI) medium and rice plant regeneration (RPR) medium were established. The RCI medium is composed of agar-solidified MS salts, B5 vitamins, 30.0 g/L sucrose, 30 g/L sorbitol, 1.0 mg/L 2,4-dicholorophenoxy

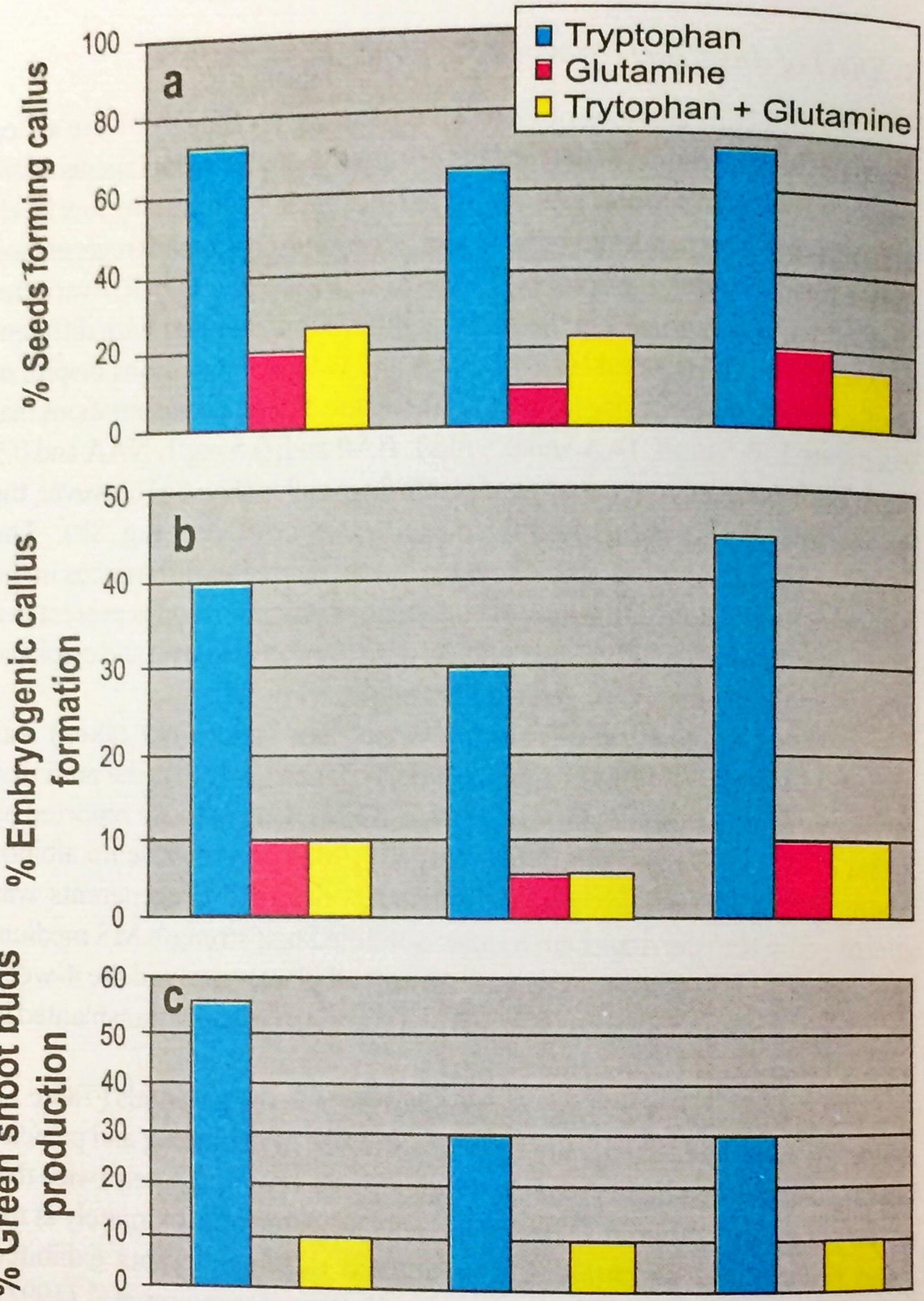

Fig. 5. Initiation of callus from upland rice seeds (a), embryogenic callus formation (b), and green shoot buds production (c) as affected by tryptophan and α-glutamine supplement in the medium.

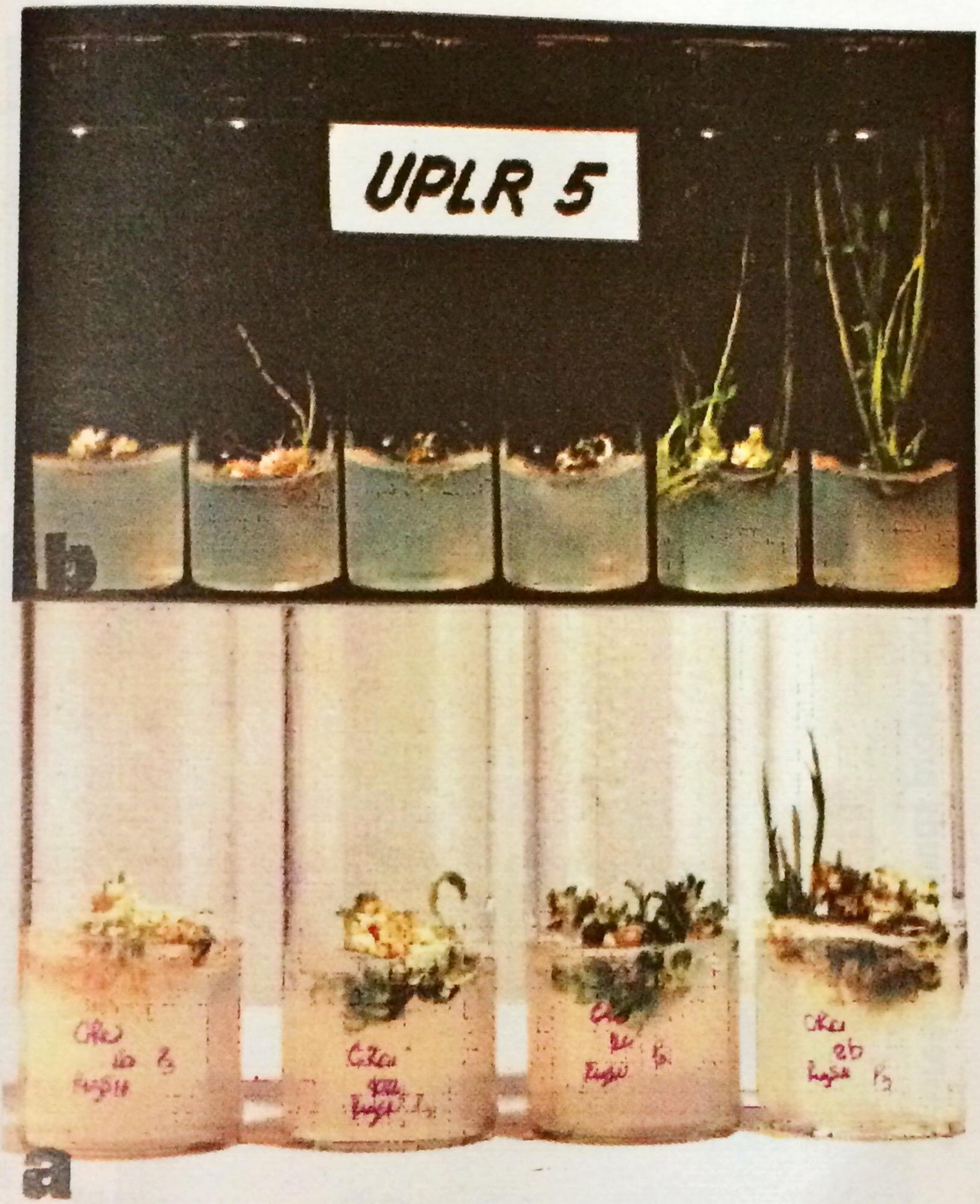

Fig. 6. Sequential stages depicting the initiation of embryogenic callus, formation of green shoot buds and regeneration of plants from mature dehulled seeds of upland rice varieties PSB Pc1 and UPLR5.

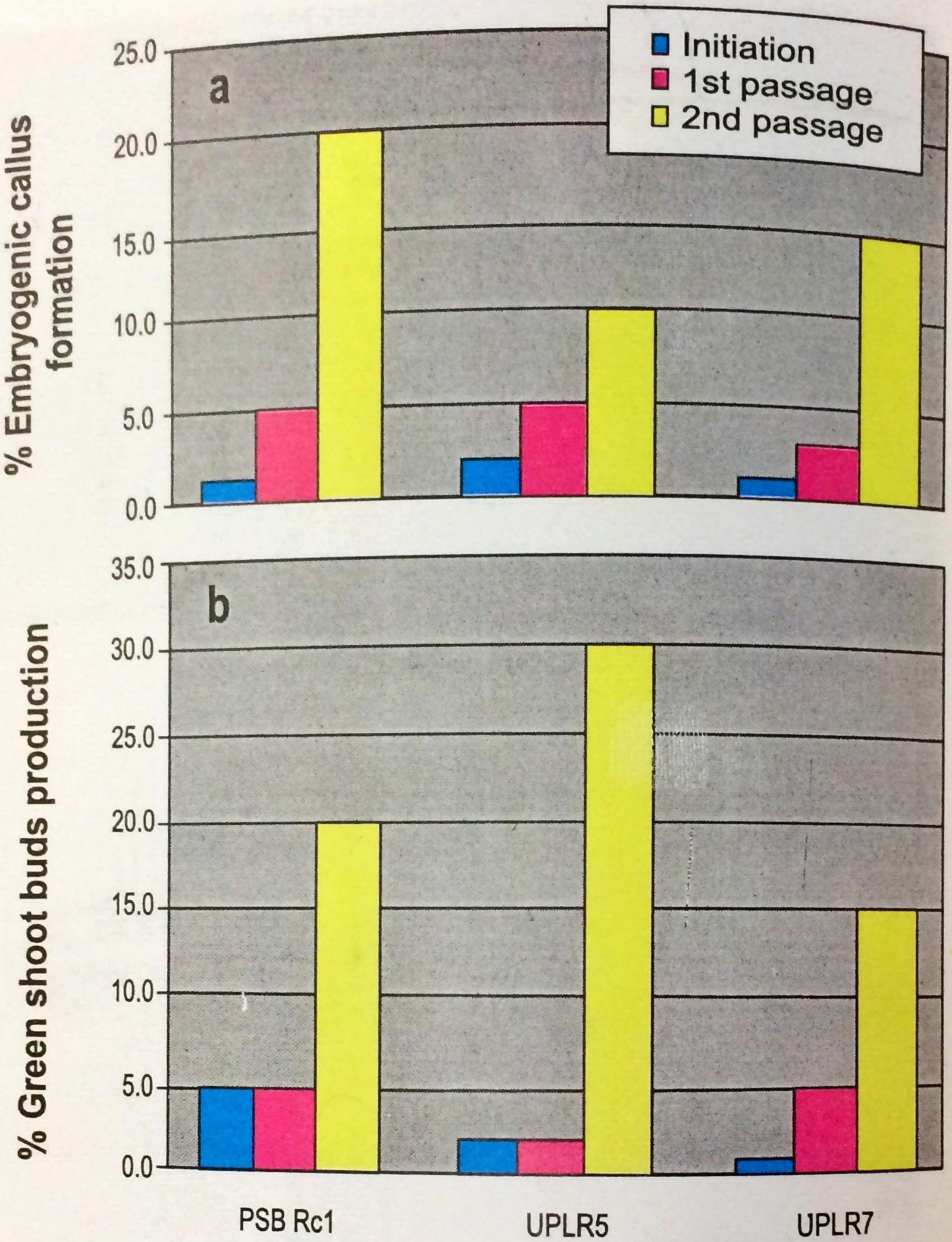

Fig. 7. Formation of embryogenic callus (a) and green shoot buds (b) from mature dehulled seeds of upland rice as influenced by the abscisic acid supplement at different culture stages

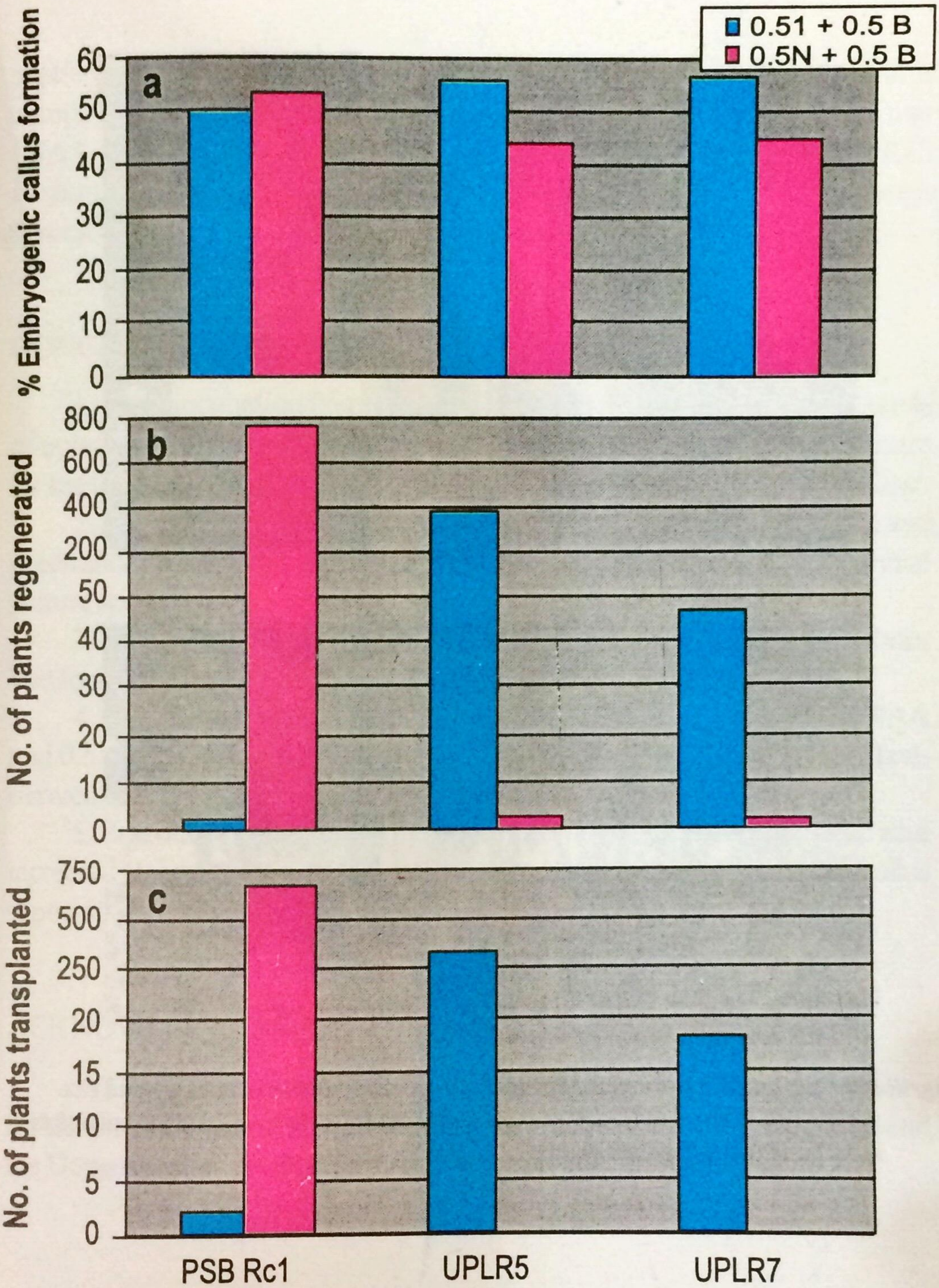

Fig. 8. Percent embryogenic callus formation (a), number of regenerated plants (b) and, number of plants transplanted in the soil (c).

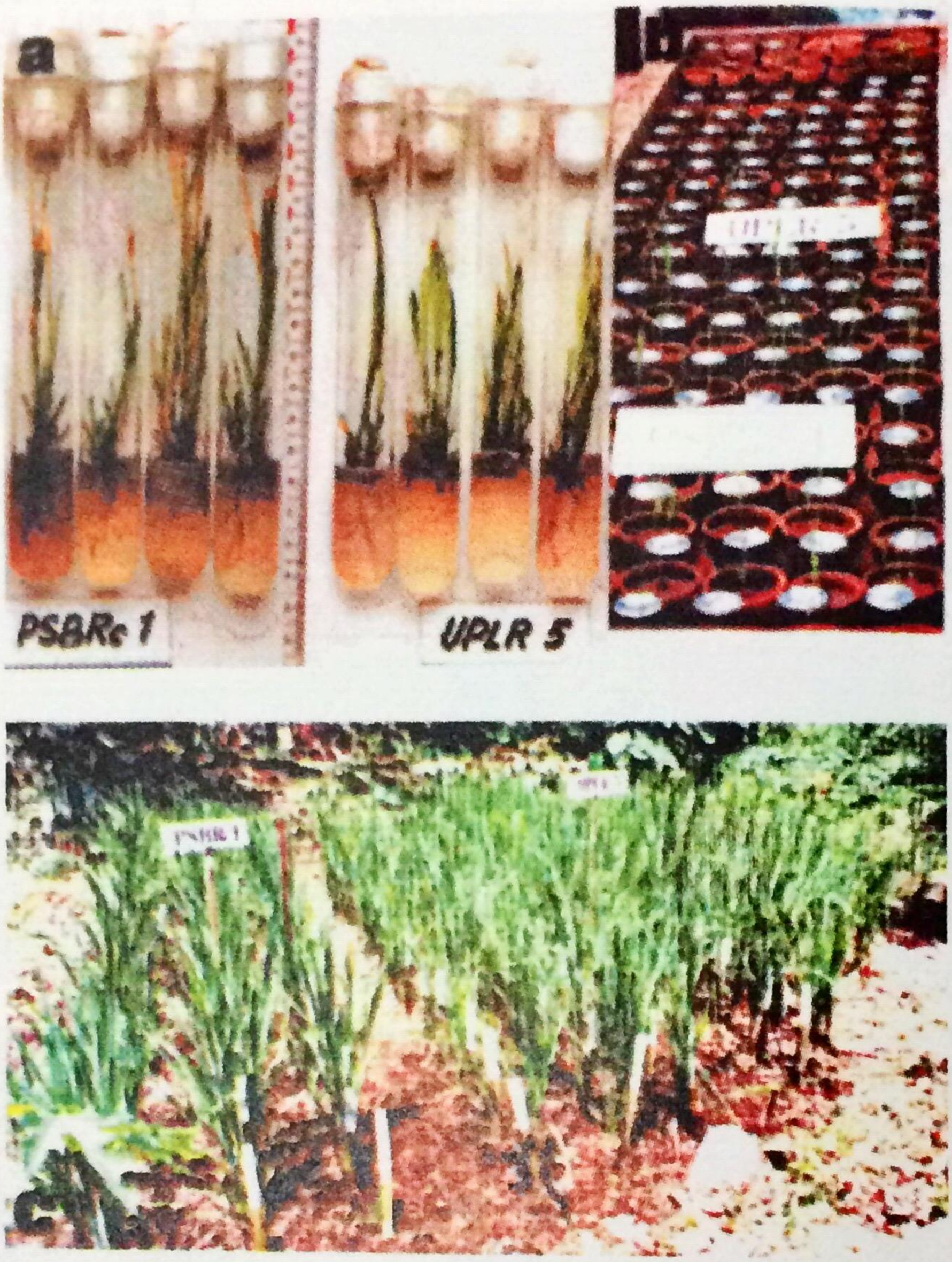

Fig. 9. Rooting, hardening and establishment of callus-derived plants of upland rice varieties PSB Rc1 and UPLR5. (a) *in vitro* rooted plantlets, (b) hardened plantlets in soil,and (c) established plants in the field.

acetic acid (2,4-D) and 0.5 mg/L kinetin at pH 5.8. The RPR medium is composed of agar-solidified MS salts, B5 vitamins, 30.0 g/L sucrose, 0.5 mg/L IAA and 0.5 mg/L BAP and 50 mg/L tryptophan. A preculture in RCI medium containing abscisic acid at the third passage or 7th week of culture is favorable for increased regeneration frequency.

CONCLUSIONS

1. Light incubation favored callus initiation from mature dehulled seeds of upland rice. However, this was no longer critical in the subsequent culture for the formation of embryogenic callus type except for the variety 'Magkaling'.

2. Sorbitol (30 g/L) promoted the formation of embryogenic callus and green shoot buds when added in the rice callus initiation medium at the initial culture stage.

3. Tryptophan at 50 mg/L enhanced embryogenic callus and green shoot bud formation but not when combined with 800 mg/L glutamine.

4. Combinations of 0.5mg/L IAA and 0.5 mg/L BAP or, 0.5 mg/L NAA and 0.5 mg/L BAP were effective for the regeneration of plants from seed-derived callus.

5. Preculture of rice callus in medium containing 1.0 mg/L abscisic acid increased plant regeneration through enhanced formation of embryogenic callus type and green shoot buds.

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