Influence of Ammonium Nitrate on Plant Regeneration in Indica Rice (Oryza sativa Linn.)

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Received 5 November 2001; accepted 3 June 2002

The effect of ammonium nitrate (an essential macrosalt) on plant regeneration from mesocotyl segments of indica rice (Oryza sativa Linn. cv. Safari-I7) has been investigated. Callus developed from the cut ends of mesocotyl segments on MS medium supplemented with 2,4-D (20 μM), BAP (2.2 μM) and various concentrations of ammonium nitrate (10-80 mM). Highly organised and nodular callus developed after transfer of morphogenic calli to the MS medium containing 5 μM 2,4-D, 2.2 μM BAP and ammonium nitrate (20-80 mM). The presence of ammonium nitrate was found to be essential for the induction and proliferation of embryogenic callus. Shoot differentiation occurred after transfer of calli to the MS medium containing 10, 20 (MS original), 40 and 80 mM ammonium nitrate alone or in combination with 8.8 μM BAP. No shoot regeneration occurred in the absence of ammonium nitrate. The highest frequency of shoot regeneration and average number of shoots per callus clump was obtained on MS medium containing 40 mM ammonium nitrate. No significant difference in regeneration frequency and average number of plantlets per callus clump was observed when BAP (8.8 μM) was incorporated in the regeneration medium containing various levels of ammonium nitrate. Regenerated shoots were rooted on MS medium containing 4.9 μM IBA. Rooted plantlets were established to soil. The results show that ammonium nitrate play an important role in plant regeneration in indica rice.

Keywords: Oryza sativa, mesocotyl segments, ammonium nitrate, morphogenic callus, plant regeneration

Introduction

Plant regeneration has been achieved from cells, tissues or protoplasts in a number of economically important crop species including rice (Narayanaswamy, 1994; Bhojwani & Razdan, 1996). Various factors like basal medium, plant growth regulators, age of the explant, genotype and partial desiccation have been reported to increase regeneration frequency in Graminaceous species (Rance et al., 1994; Bregitzer et al., 1995; Oinam & Kothari, 1995; Jain et al., 1996). In recent years, it has been realised that cereal tissue culture requires high concentrations of macro and microelements in order to achieve high frequency plant regeneration (He et al., 1989; Dahleen, 1995; Bregitzer et al., 1998; Sahrawat & Chand, 1999). Preece (1995) suggested that it is possible to partially substitute plant growth regulators in the basal medium by organic and inorganic nutrients. In wheat, high frequency embryogenic callus and development of embryos has been achieved by doubling the concentration of macrolelements of MS medium (Ozias-Akins & Vasil, 1983; He et al., 1989). Carman et al. (1988) compared several media and reported that the media containing double concentrations of macroelements of MS medium produced more embryogenic protuberances than the other media used in the experiment. Khanna & Raina (1997) and Podder et al. (1997) reported improved plant regeneration in indica rice and finger millet, respectively by optimizing the concentrations of macroelements. Also, regeneration of plants and subsequent growth is influenced by nitrogen, its form and ammonium to nitrate ratio (Lillo, 1989). These studies revealed that macroelements might play an important role as a catalyst in improving plant regeneration frequency.

No attempt has been made so far to investigate the effect of an individual macroelement for high frequency embryogenic callus induction and plant regeneration in indica rice. The present study deals with the effect of the ammonium nitrate (an essential macrosalt) on morphogenic callus initiation, regeneration frequency and absolute number of regenerated plantlets per culture. Such studies are useful in genetic transformation of indica cultivars of
Fig. 1—Embryogenic callus induction and plant regeneration from mesocotyl segments of indica rice (*Oryza sativa* L. cv. Safari-17): A, Callus formation from mesocotyl segments after four weeks of culture on MS medium supplemented with 20 μM 2,4-D, 2.2 μM BAP and 40 mM ammonium nitrate; B, Organised and nodular calli formation after six weeks of transfer to MS medium containing 5 μM 2,4-D, 2.2 μM BAP and 40 mM ammonium nitrate; C, Shoot buds induction two weeks after transfer of embryogenic and nodular calli to MS medium containing 40 mM ammonium nitrate; D, Multiple shoots formation six weeks after transfer of embryogenic calli to MS medium containing 40 mM ammonium nitrate; E, Regenerated shoots rooted on MS medium containing 4.9 μM IBA & F, Regenerated plants in pots.
Materials and Methods

Explant Source and Preparation of the Explants

Mature seeds of indica rice (Oryza sativa Linn. cv. Safari-17) were collected from Indian Agricultural Research Institute, New Delhi, India. The seeds were dehusked and surface sterilised in 0.1% (w/v) aqueous solution of mercuric chloride for 10 min followed by quick dip (30 sec) in 70% (v/v) ethanol and then rinsing five times in sterile distilled water. An average 3-4 sterilised seeds were aseptically inoculated in 25 x 150 mm glass tubes (Borosil, Mumbai, India) containing Murashige and Skoog (1962) medium and 3.0% (w/v) sucrose. The cultures were kept under 16/8-hrs light/dark photoperiod provided by cool-white fluorescent light (irradiance 50 μmol m⁻² s⁻¹), temperature being 24±2°C. Mesocotyl segments (5-6 mm) were dissected aseptically on 6th day from in vitro grown seedlings. Mesocotyl segments were used as explants.

Culture Initiation

Mesocotyl segments were cultured on MS (1962) medium supplemented with 2,4-D (20 μM), BAP (2.2 μM), sucrose (3%, w/v) and different concentrations of ammonium nitrate (0, 10, 20 (MS original), 40 and 80 mM) for obtaining embryogenic calli. The effect of ammonium nitrate was studied by changing the concentration of the salt while the concentration of other salts were maintained as that of MS (1962) basal medium. The pH of the medium was adjusted to 5.8 using 0.1 N NaOH or HCl before autoclaving. Routinely, 25 ml of the molten medium gelled with 0.8% (w/v) agar was dispersed into culture tubes (25x150 mm) and plugged with non-absorbent cotton wrapped in one layer of cheese-cloth. The culture tubes were then steam sterilised at 121°C and 104 kPa for 15 min. The cultures were incubated under 16/8-hrs light/dark photoperiod (provided by cool-white fluorescent light, irradiance 50 μmol m⁻² s⁻¹) at 24 ± 2°C and relative humidity 60%. After 6 weeks, mesocotyl segments derived calli were transferred to maintenance medium (medium having same composition as used for callus induction except that concentrations of 2,4-D reduced to 5 μM) for another six weeks (one subculture period was of three weeks).

Plant Regeneration

For shoot differentiation, morphogenic calli were transferred to MS medium supplemented with 0, 10, 20, 40 or 80 mM ammonium nitrate alone or in combination with 8.8 μM BAP. Regeneration frequency was scored four weeks after transfer of morphogenic calli onto regeneration medium. Average number of shoots per callus clump was scored eight weeks after transfer of calli to the regeneration medium. For rooting, shoots (reaching a length of 3 to 4 cm) individually or in clumps (2 to 3 shoots) were transferred to rooting medium, MS containing 4.9 μM IBA. Well-developed plantlets with good root system were successfully transferred to pots.

Statistical Analysis

The student t-test was applied to determine significant difference for plant regeneration. P values (<0.05) were considered significant.

Results and Discussion

Callus Induction

Initiation of callus occurred from cut ends of mesocotyl segments within two weeks of inoculation on MS medium containing 20 μM 2,4-D, 2.2 μM BAP and different levels of ammonium nitrate (10-80 mM). Initially yellowish, nodular calli developed from the cut ends of mesocotyl segments, which during subculture covered the entire surface of the explants (Figs 1A, B). After six weeks of culture initiation, morphogenic calli were transferred to MS medium supplemented with 5.0 μM 2,4-D, 2.2 μM BAP and various concentrations of ammonium nitrate, 0, 10, 20 (control, MS original), 40 or 80 mM. Highly organised and nodular calli with numerous smooth surface protuberances developed within three weeks of subculture. Formation of morphogenic calli was influenced by ammonium nitrate concentrations incorporated in the MS medium. Maximum morphogenic and nodular callus developed on MS medium containing 5.0 μM 2,4-D, 2.2 μM BAP and 40.0 mM ammonium nitrate. While no embryogenic callus formation was seen on MS medium devoid of ammonium nitrate.

Plant Regeneration

Shoots regeneration occurred after transfer of morphogenic calli to MS medium supplemented with different concentrations (0, 10, 20, 40 or 80 mM) of ammonium nitrate alone or in combination with BAP (8.8 μM). Formation of green patches on callus tissues occurred six days after transfer of morphogenic calli to MS medium enriched with 40 mM ammonium
nitrate, while green patches formed after 12-15 days of transfer of calli to MS medium containing 10, 20 or 80 mM ammonium nitrate. During subculture, these green patches converted into tiny shoot buds (Fig. 1C) and finally proliferated into multiple shoots (Fig. 1D). Shoot regeneration frequency and average number of regenerated plantlets were found to be correlated with the concentration of ammonium nitrate added to the regeneration medium (Fig. 2). Highest frequency (95.2%) of shoot regeneration occurred when morphogenic calli were transferred to MS medium containing 40.0 mM ammonium nitrate. Response was significantly low on MS medium containing 10, 20 or 80 mM ammonium nitrate (Fig. 2). A positive correlation was also observed between the concentrations of ammonium nitrate added in the medium and average number of shoots regenerated per callus clump (Fig. 2). Maximum average number of shoots (14.3 ± 0.8) was observed on MS medium containing 40 mM ammonium nitrate. No considerable difference in regeneration frequency and average number of plantlets per callus clump was observed when BAP (8.8 μM) was added in the MS medium. No shoot bud formed on MS medium devoid of any concentration of ammonium nitrate. However, when 8.8 μM BAP was added in the MS medium devoid of ammonium concentration, regeneration frequency increased from 0 to 14.1%. Root differentiation occurred after transfer of healthy shoots on MS medium containing 4.9 μM IBA (Fig. 1E). Regenerated plantlets with well-developed root systems were transferred to pots where they grew well (Fig. 1F).

The results of the present study show that the concentrations of ammonium nitrate of MS medium affects morphogenic callus formation frequency, differentiation potential of callus and average number of regenerated plantlets per callus clump. Ammonium nitrate has been found to be an essential macroelement for the formation and growth of embryogenic callus from mesocotyl segments of indica rice. On omitting ammonium nitrate from the medium, neither induction of embryogenic callus nor subsequent regeneration was observed. Frequency of morphogenic callus formation was constantly increased with the increased concentration of ammonium nitrate from 10 to 40 mM.

In recent years, high frequency morphogenic callus induction and regeneration frequency has been achieved in Gramineous species by optimising the concentrations of inorganic macronutrients (He et al., 1989; Khanna & Raina, 1997; Podder et al., 1997) and micronutrients (Dahleen, 1995; Bregitzer et al., 1998; Sahrawat & Chand, 1999) in basal medium. Appropriate manipulation of the nutrient content especially the nitrogen sources have been reported to stimulate embryogenesis in cereals (Halperin & Wetherell, 1965; Meijer & Brown, 1987). In wheat, a double strength MS medium together with casein hydrolysate and other adjuvants supported an increase (30%) in the induction of embryogenic calli (Oziash-Akins & Vasili, 1983). He et al. (1989) reported high frequency embryogenic callus induction in wheat by increasing two to three times concentrations of macroelements of MS medium. Kohlenbach (1978) observed a direct effect of auxin and nitrogen on embryogenesis.

Highest frequency of shoot regeneration and average number of shoots per callus clump was observed on MS medium containing 40 mM ammonium nitrate. Whereas response was comparatively low on MS medium containing 10, 20 or 80 mM ammonium nitrate. Plantlets regenerated in the presence of higher concentrations of ammonium nitrate were dark green in colour and healthy as compared with those obtained on medium containing normal (20 mM) levels of ammonium nitrate. Khanna & Raina (1997) have shown significant enhancement in plantlets regeneration frequency on higher

![Fig. 2—Shoot regeneration frequency and average number of plantlets from mesocotyl segments derived calli of indica rice (cv. Safari-17) on MS medium containing various concentrations (10.0-80.0 mM) of ammonium nitrate. Evaluation eight weeks after transfer of embryogenic calli to regeneration medium. Vertical bars represent standard error. Asterisks represent significant difference (P< 0.05) from control (MS medium containing 20 mM ammonium nitrate).]
concentrations of potassium nitrate and ammonium sulphate in indica rice. They showed that basmati indica rice (cv. Karnal local) required KNO₃ and (NH₄)₂SO₄ instead of KNO₃ and NH₄NO₃ in the basal medium for maximum regenerability of callus. They observed cent per cent regeneration frequency when 35 mM of KNO₃ and 5 mM of (NH₄)₂SO₄ was added in the medium as compared to 65.0% regeneration frequency on MS medium containing 19 mM KNO₃ and 21 mM NH₄NO₃. The modification in MS medium resulted in 6-7 regenerants per regenerating calli as compared to 2-3 regenerants on MS medium.

Podder et al. (1997) have reported high frequency plant regeneration from embryogenic calli of *Eleusine coracana* by optimising the concentrations of ammonium nitrate in MS basal medium. They obtained maximum number of plantlets per callus clump on MS medium containing 80 mM ammonium nitrate without any growth regulators. Species-specific nitrogen requirements have been described for a wide range of different culture systems and the ratio of NH₄⁺/NO₃⁻ was often found to be important (Bellini et al., 1990; Selby & Harvey, 1990).

The above findings indicate that high frequency plantlets regeneration can be achieved in indica rice by optimising the concentrations of ammonium nitrate in the medium.

References


