RESEARCH ARTICLE

Somatic embryogenesis and plant regeneration from male flower buds in banana

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Embryogenic cultures were established using young male flowers in five cultivars of banana (*Musa* spp.). Embryogenic callus continued to rapidly proliferate on MS medium supplemented with 0.22 µM BA and 1.14 µM indole-3-acetic acid (IAA) and somatic embryos developed during subcultures on the same medium. Green plumule emerged from the embryos followed by the development of roots within a span of 6 to 8 weeks upon transfer to ½ strength Murashige and Skoog medium (MS) + 0.5 g/l malt extract (ME) and 0.1% activated charcoal (AC) and subsequently developed into complete plantlets. Normal plant development was noticed following transplantation into paper cups in the greenhouse.

BANANAS and plantains are one of the world's major fruit crops and staple food for millions of people. In India, banana ranks fourth in terms of gross value after paddy, wheat and milk and is the largest fruit crop, in terms of production. Majority of the edible bananas are triploid and are propagated vegetatively by suckers. In addition to this, sterility and polyploidy often hamper the breeding programmes for the development of superior banana varieties.

Plant cell and tissue culture techniques have helped in the rapid multiplication of elite varieties employing shoot tips or floral apices[1]. Somatic embryogenesis has been reported from leaf sheath or rhizome fragments from *in vitro* plants[2], thin sections from proliferating buds[3], immature zygotic embryos[4] and male flower bud cultures[5,6]. Somatic embryogenesis leading to embryo production and plant regeneration offers advantages for mass propagation of elite cultivars and also provides a useful system for genetic manipulation[7,8]. In the present communication, we report on the initiation and maintenance of highly proliferating embryogenic callus and plant regeneration in some elite Indian banana cultivars.

Materials and methods

Experimental plant material included cvs. Rasthali (AAB), Basrai (AAA), Shreemanti (AAA), Lokhandi (AAA ?) and Trikoni (AAA ?). Male inflorescences collected from the field-grown plants, were surface cleaned with absolute alcohol. Under a sterile laminar air flow, the outer enveloping bracts were removed and the inner part (2–3 cm in length) containing male flower primordia (from 0 to 15, 0 being the meristem) was isolated. Individual male flower primordia were cultured on Murashige and Skoog[9] medium (MS) or White's[10] medium supplemented with 18.10 µM 2,4-dichlorophenoxy acetic acid (2,4-D), 5.37 µM napthalene acetic acid (NAA), 5.71 µM indole 3-acetic acid (IAA) and 4.09 µM *d*-biotin, 3% sucrose and 0.2% gelrite for callus induction. For the proliferation of callus and somatic embryo induction, a medium supplemented with 0.22 µM benzylaminopurine (BA) and 1.14 µM IAA, 3% sucrose and vitamins[11] was used. Callus was also cultured on media supplemented with mannitol (3%), to study its effect on embryogenic callus development. The pH of all the media was adjusted to 5.7 prior to autoclaving and all the cultures were maintained in an incubation room at 25 ± 2°C and 12 h photo-period with 1000 lux.

For conversion of somatic embryos into plantlets, ½ strength MS medium supplemented with 0.5 g/l malt extract (ME) and 0.1% activated charcoal (AC), Morel's vitamins, 3% sucrose and 0.2% gelrite were employed. Fully grown plantlets with good shoot and root system were then transplanted into paper cups with soilrite and then into polybags in the greenhouse.

Results and discussion

Male flower buds cultured on MS or White's medium supplemented with 18.10 µM 2,4-D, 5.37 µM NAA, 5.71 µM IAA and 4.09 µM *d*-biotin, showed enlargement of the floral primordia and the development of whitish embryogenic callus, after 2 to 3 months of inoculation (Figure 1 *a*). MS medium was found to be better than the White's medium (41.6%) for embryogenic callus (66.5%). The embryogenic callus showed several developed embryos on the surface and the smear preparations of the callus tissue showed early stages of embryo development (Figure 1 *b*). The embryogenic tissues showed repeated proliferation during subcultures on MS + 0.22 µM BA + 1.14 µM IAA, resulting in an increase by 8.07-fold callus fresh weight for each

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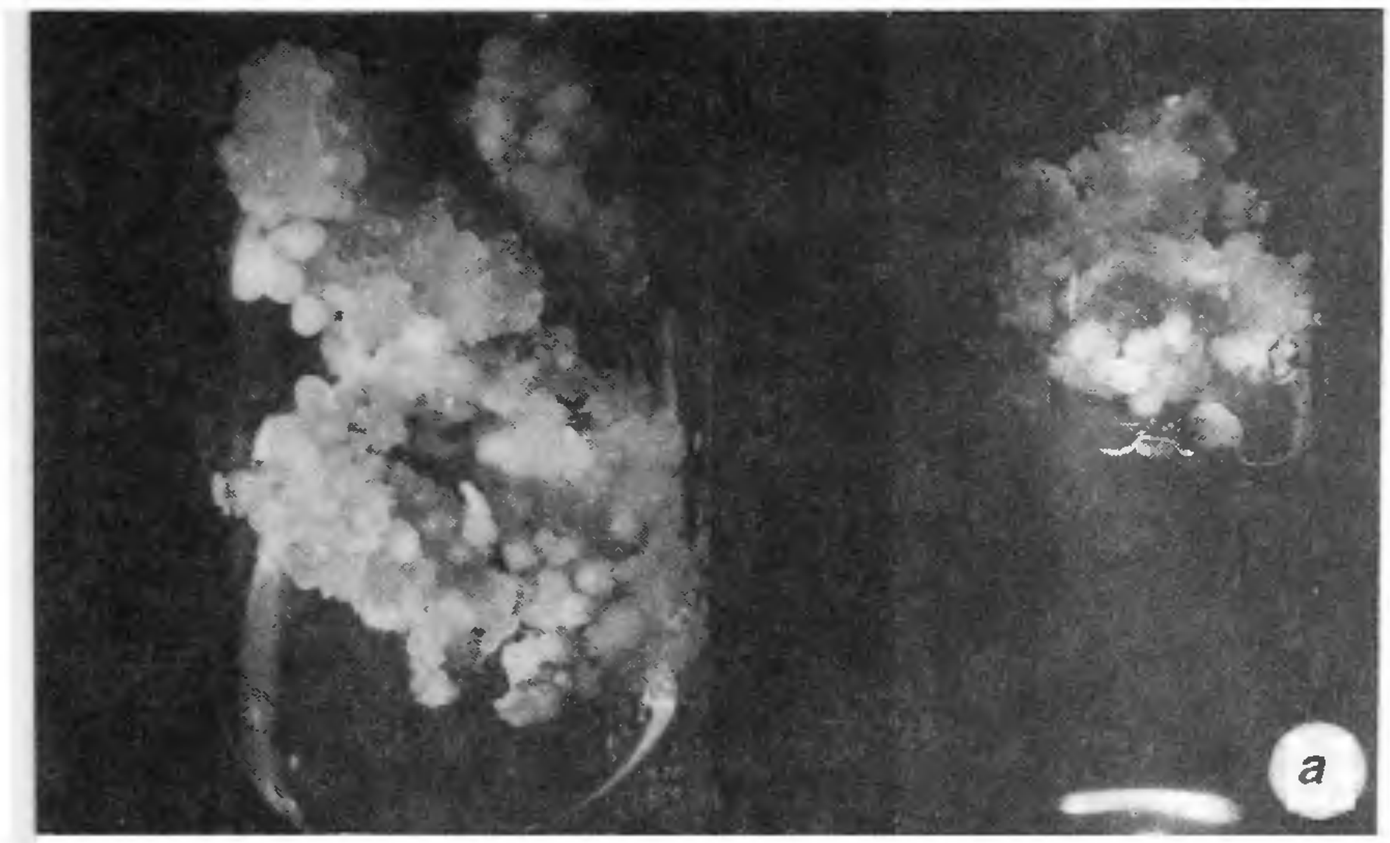
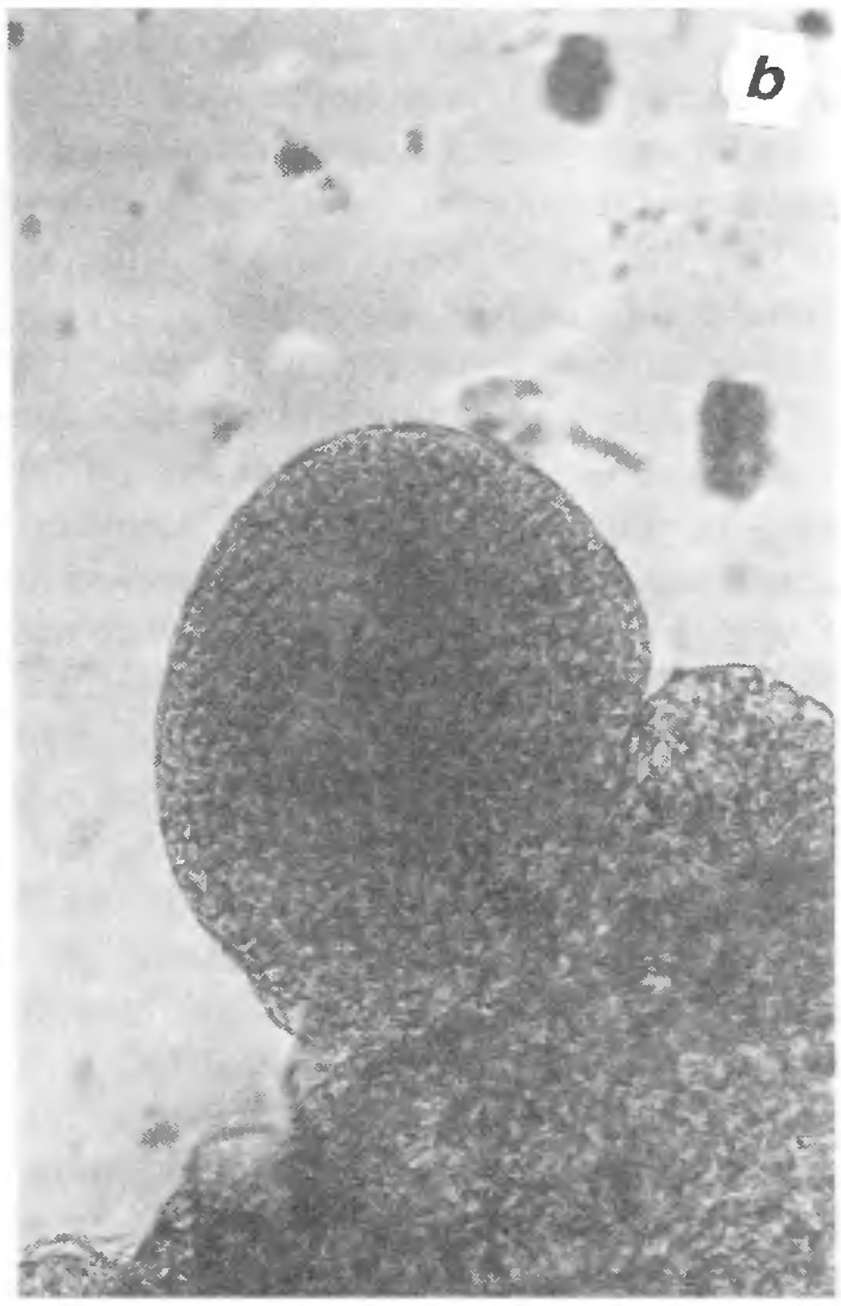
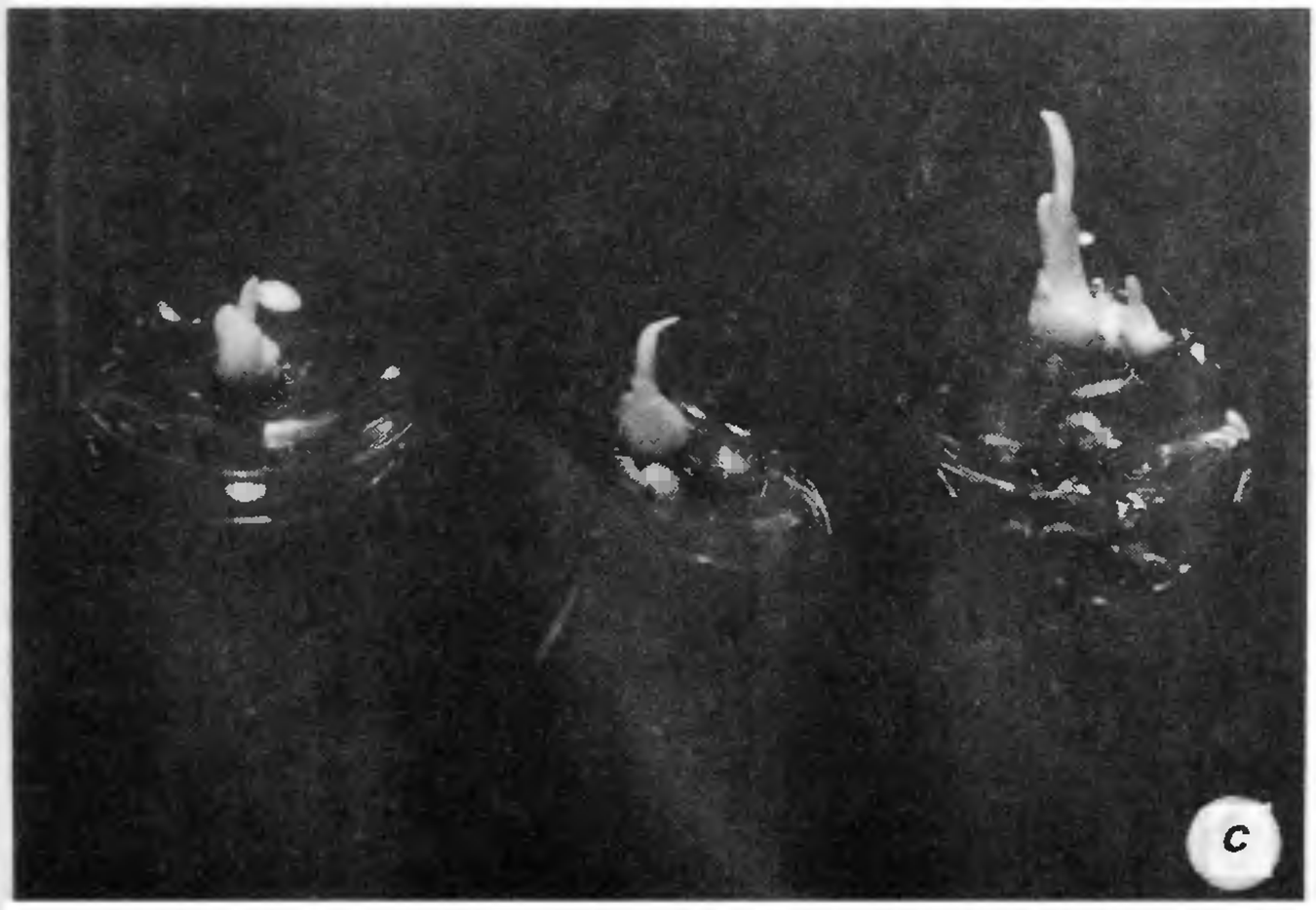
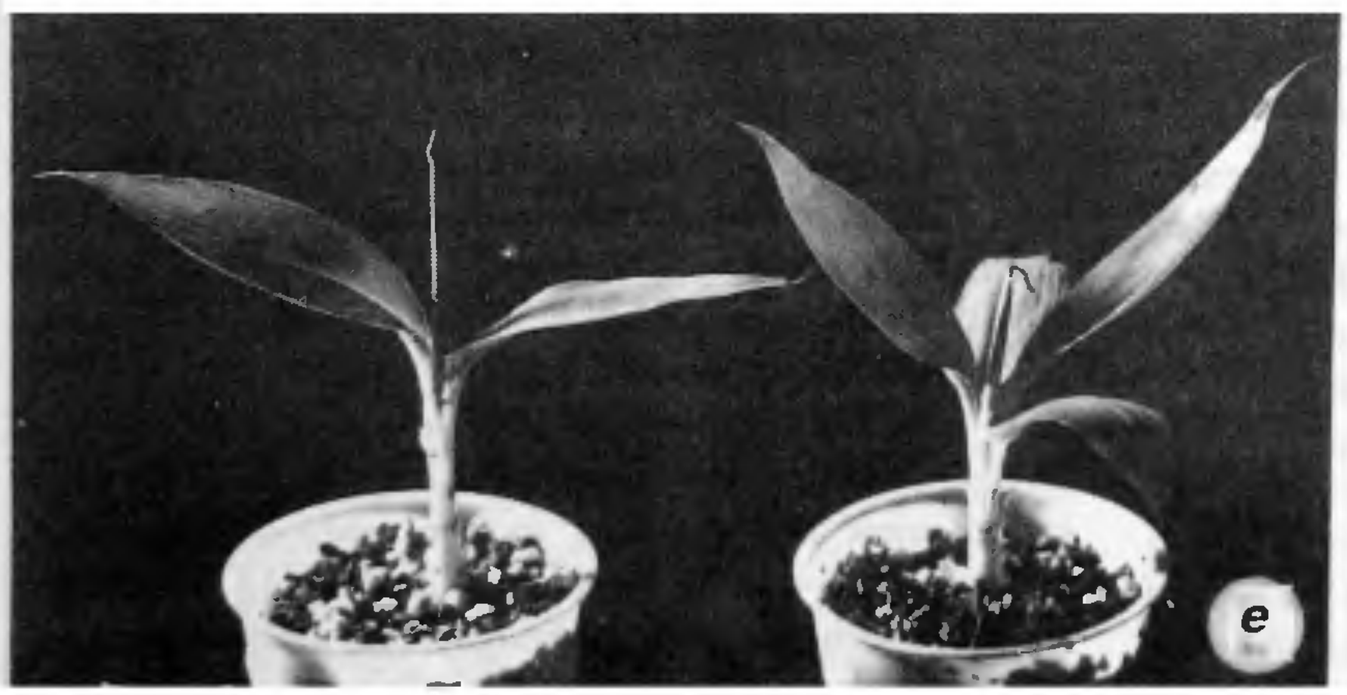








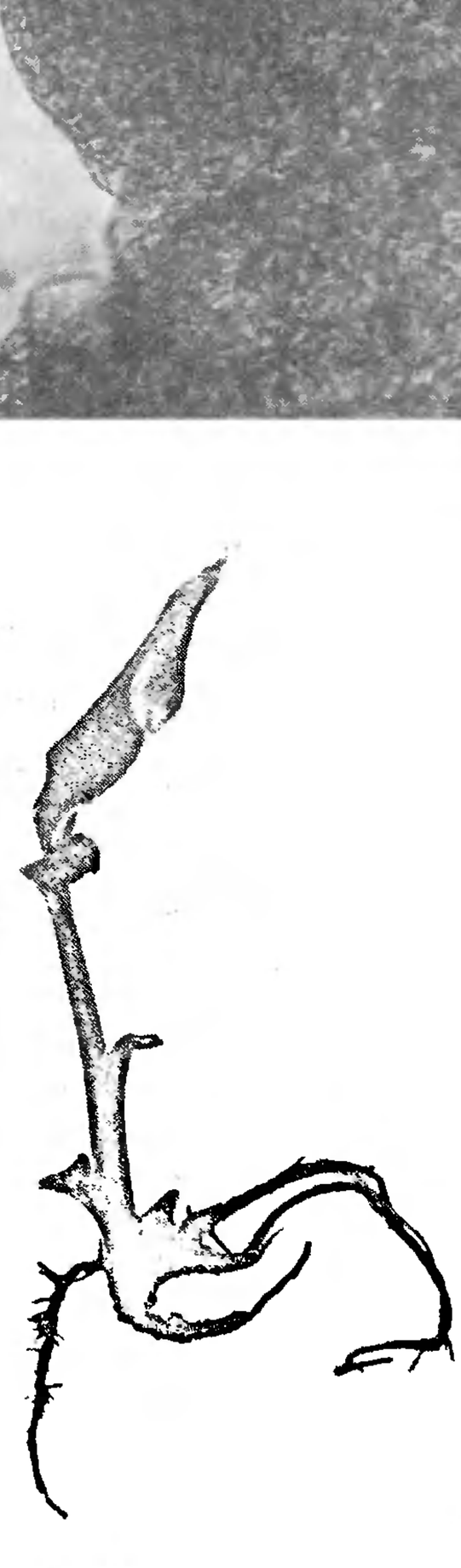


Figure 1 ***a–e.*** Somatic embryogenesis and plant regeneration from male flower buds of banana cv. Rasthali. ***a***, Embryogenic callus cultures from young male flower buds on MS medium with 0.22 µM BA and 1.14 µM IAA; ***b***, Squash preparation of embryogenic callus showing globular stage embryo; ***c***, Germination of somatic embryos on 1/2 MS medium + 0.5 g/l ME and 0.1% AC; ***d***, A somatic embryo derived plantlet with good root system; and ***e***, Regenerated plants in paper cups with soilrite in the greenhouse.

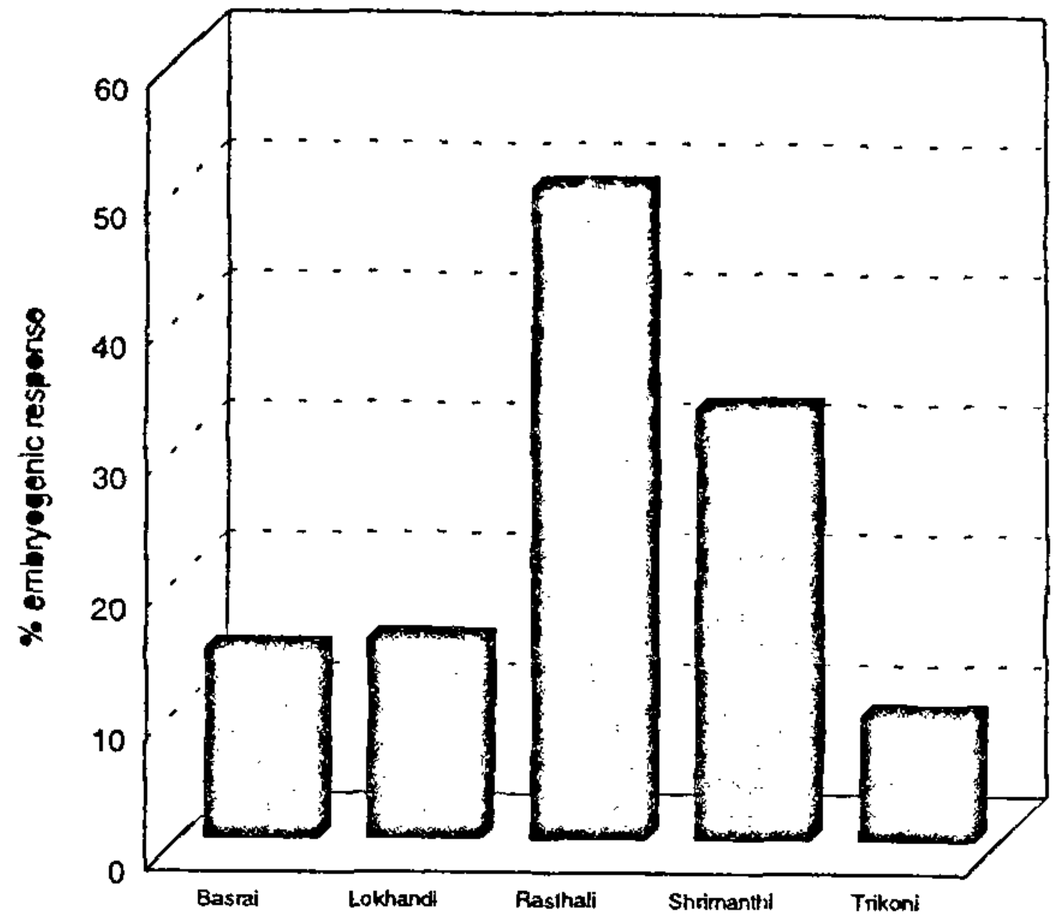
passage of four weeks. Embryogenic callus cultured on MS medium + 0.22 μM BA + 1.14 μM IAA with either 3% sucrose or 3% mannitol showed differences in callus morphology. The medium with 3% sucrose induced rapid proliferation of semi-friable embryogenic callus (90–95%), while with mannitol, the callus became nodular and compact (60–65%).

Five cultivars tested for embryogenic callus formation showed differential culture response (Figure 2). Rasthali was found to be highly responsive (50%) followed by 30% in Shreemanti. The other cultivars Basrai, Lokhandi and Trikoni were less responsive (8–15%). Embryogenic cultures of cv. Rasthali were maintained for a period of over 2 years without any decline in the capacity for proliferation. For the conversion of somatic embryos, the MS medium supplemented with 0.1% yeast extract, 1 g/l caesin hydrolysate (CH) and 1 g/l maltose was tested, either with agar or gelrite as the gelling agent. While the medium gelled with gelrite (0.2%) induced the formation of compact callus and globular embryos, the medium with agar (0.8%) showed > 80% browning of cultures.

Somatic embryos developed into plantlets (5–8%) on ½ strength MS basal medium without any growth regulators supplemented with 0.5% ME + 0.1% AC and gelled with 0.2% gelrite. Green plumule emerged from the embryos followed by the development of roots (Figures 1 *c*, *d*) within a span of 6 to 8 weeks. Plantlets developed normally when transplanted into paper cups with soilrite (Figure 1 *e*) in the greenhouse and 24 well-grown plants were transferred to the field.

Young floral tissues are known to be conducive to *in vitro* culture with good embryogenic potential[12]. In the present study, young male flower primordia of banana were used for the induction of embryogenic callus and somatic embryos which required different hormonal treatments. Inductive treatments included three auxins (IAA, NAA and 2,4-D) while a cytokinin with auxin (BA + IAA) was necessary for embryogenic callus proliferation and somatic embryo development. Navarro *et al.*[6] also noted that three auxins were essentially required for induction. Among the cultivars with different genomic backgrounds, Rasthali (AAB) showed good embryogenic response over Basrai (AAA) and Shreemanti (AAA) and others with AAA genome. A similar genotypic difference in culture response was observed in cultivars with AAA, AAB and ABB genomic status[5].

In the present study, embryogenic callus exhibited rapid proliferation on a medium with BA + IAA, with embryos developing on the callus surface. Several embryogenic cell clumps and globular stage embryos were observed while some developed into somatic embryos. Escalant *et al.*[5] also observed white, translucent callus and somatic embryos in cultures of young male flowers of cv. Grande Naine (AAA). Multiplication and maintenance of embryogenic cultures was achieved by culturing somatic embryos in a temporary immersion system, while Novak *et al.*[2] and Dheda *et al.*[3] described a liquid phase for the development of somatic embryos. In our studies, however, a liquid phase for somatic embryogenesis was not essential. Inclusion of mannitol (in addition to sucrose) in the medium is known to induce osmotic stress and embryogenic cultures were obtained in rice[13]. However, in this study, with the inclusion of mannitol, primary callus became nodular and more compact than on the sucrose medium.



An average of 96 cultures obtained from four replications of 24 each.

Figure 2. Frequency of embryogenic callus formation from young male flower buds in different cultivars of banana.

The development of an *in vitro* regeneration system is essential for studies related to genetic manipulation of banana. The embryogenic culture system described in this paper demonstrates the potentiality of the male flower bud explants for use in raising highly proliferative, embryogenic cultures for banana cultivars. Cultivar Rasthali, responding better *in vitro*, has fruits with sweet taste and thin peel but is highly susceptible to *Fusarium* wilt. Developing protocols for *in vitro* plant regeneration may aid efforts in the selection of disease-resistant clones. Separate media manipulations for embryo maturation were not required as the embryos developed into plantlets on the same medium making the culture system much simpler. Although, the plant conversion rates are low, we believe that further refinement in the media would lead to enhanced embryo to plant conversion frequency. Further exploitation of the system of somatic embryogenesis for mass propagation, artificial seeds and for generating transgenic plants may provide a significant step in the improvement of banana.

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RESEARCH COMMUNICATIONS

Cytochrome P-450 in drug-resistant *Mycobacterium tuberculosis*

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Methods for the isolation and spectrophotometric determination of cytochrome P-450 in mycobacteria were standardized. Cytochrome P-450 levels were estimated in *Mycobacterium tuberculosis* organisms sensitive to both isoniazid and rifampicin and resistant to any of the two drugs. Cytochrome P-450 was isolated and its presence was shown in *M. smegmatis*, *M. fortuitum*, *M. chelonae* and *M. tuberculosis* $H_{37}Rv$. The cytochrome P-450 content was significantly elevated in *M. tuberculosis*, resistant to both isoniazid and rifampicin when compared with the corresponding sensitive strains. It therefore appears that cytochrome P-450 might play a role in causing drug resistance in tuberculosis.

TUBERCULOSIS is one of the most alarming infectious diseases facing the world today, particularly with the advent of multi-drug resistant organisms. Bacteria develop resistance to drugs via a limited number of mechanisms[1], some of which apply to mycobacterial drug resistance[2] and this appears to be a multi-locus phenomenon and several factors contribute to the development of resistance. The role of drug-deactivating enzymes in the development of drug resistance is well established in bacteria[3] and insects[4]. This phenomenon usually involves increased activity of a particular enzyme responsible for degrading the active drug.

Cytochrome P-450, earlier referred to as the CO-binding pigment, was first described by Klingenberg[5]. It belongs to a group of hemoproteins and forms a vital component of the mixed-function oxidase system of the endoplasmic reticulum. Cytochrome P-450 constitutes the most powerful oxidizing enzymes and is involved in the metabolism and biotransformation of a wide variety of drugs and endogenous compounds. It is found in abundance not only in the hepatic microsomes, but also in the microsomes and mitochondria from the adrenal cortex. The hemoprotein is found in liver microsomes of a variety of vertebrates, including mammals, birds, snakes, frogs and fishes, plants, bacteria[6], bacteroids[7] and yeast[8].

An association between drug resistance and cytochrome P-450 drug-metabolizing enzyme activity has been reported in certain species of Plasmodium[9,10], *Drosophila melanogaster*[11], *Musca domestica*[12], *Trypanosoma cruzi*[13], *Leishmania mexicana*[14], *Heliothis virescens*[15], *Lucilia cuprina*[16], certain Candida species[17–19], *Cryptococcus neoformans*[20], etc. In all these studies, it has been clearly shown that the resistant species had elevated levels of cytochrome P-450 than the corresponding sensitive ones. Indirect evidence relating to mechanism of resistance in *M. avium* with respect to quinolones has been shown to be associated with bacterial P-450 activity[21]. For example, the resistant strains might have enhanced P-450 activity in the periplasm, resulting in an increased rate of metabolism of quinolones, which, therefore, cannot reach the critical concentration needed to be effective. However, when cytochrome P-450 is inactivated, the resistance mechanism gets defeated.

Recently the complete genomic sequence of *M. tuberculosis* has been reported by Cole *et al.*[22]. A large

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