Callusing from rice root explants: Adventitious root formation precedes callus initiation response

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The phenomenon of adventitious root induction from various parts of the plant system under specific experimental conditions is widely practised. We note in this study that roots excised from 7-day-old rice seedlings when cultured on Murashige and Skoog's nutrient medium supplemented with 2,4-dichlorophenoxyacetic acid produced a large number of adventitious roots. It was further noted that the basal cells of the adventitious roots dedifferentiate to form calli masses. This response of adventitious root formation and callusing was 100% as all the tested root explants formed adventitious roots. Apart from four different cultivars of rice, three other genera namely wheat, maize and brassica showed formation of adventitious roots and calli under culture conditions. The monocot genera (rice, wheat and maize) showed more conspicuous rooting and callusing response as against brassica (a dicot). Adventitious root-derived rice calli were shown to regenerate shoot primordia with high frequency (88%) upon transfer to the same medium devoid of 2,4-dichlorophenoxyacetic acid.

It has been shown that many plant parts form adventitious roots from cutting (explant) under specific conditions which among other variables, include auxin treatment[1]. Production of adventitious roots is considered to be an important phenomenon for asexual propagation of the plants[1]. Apart from propagation, these roots can be used for numerous other applications including secondary metabolite production[2]. There is a wide range in adventitious root-forming ability in plants which can vary from those which easily root to those which do not[3]. There is no detailed study on adventitious root-forming ability in rice which is an agronomically important crop. Our laboratory has interest to establish protocols of rice tissue culture methods for the genetic transformation of this crop with agronomically important genes. For this, we are testing the callusing response of different explants from rice seedlings to different combinations of nutrient adjuvants. In the course of this search, we find that detached roots of young rice seedlings (from four different cultivars) as well as three other genera (namely wheat, maize and brassica) develop a large number of adventitious roots in Murashige and Skoog[4] medium (MS medium) supplemented with synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D). We further note that instead of developing into normal functioning roots, these adventitious roots form callus from their basal portions on the same medium. These calli regenerated shoots with high frequency in hormone-free MS medium.

Dehusked rice (*Oryza sativa* L. cvs Taipei 309, IR 54, Basmati 370 and Pusa 169) seeds were surface-sterilized using 0.1% $HgCl_2$ for 20 min, while wheat (*Triticum aestivum* cv Punjab 1) and maize (*Zea mays* cv Agaiti) seeds were surface-sterilized with the same reagent for 10 min. Brassica (*Brassica juncea* cv RLM 198) seeds were surface-sterilized using 5% detergent solution (5 min), 70% ethanol (2 min) and 4% sodium hypochlorite (9 min) with 3–4 washes after each step with sterile distilled water. The seeds were subsequently washed with sterile distilled water (4–5 washes) and kept soaked overnight and germinated in 250 ml flasks (10 seeds per flask) on MS medium supplemented with 100 mg l^{-1} myo-inositol, 0.1 mg l^{-1} thiamine-HCl and 30 mg l^{-1} sucrose. The pH of the medium was adjusted to 5.8, to which agar powder was added to final concentration of 0.8%, prior to autoclaving. The flasks containing seeds were placed in dark for two days and then transferred to light (12/12 h light-dark cycles) in a culture room at 26 ± 1°C. Callus tissues were initiated from roots employing two portions of the detached roots (from 7-day-old seedlings) namely (a) proximal to root tip portion (nearly 10 mm from the tip portion) and (b) distal to root tip (two segments of 10 mm each, excised from the roots after the proximal 10 mm is removed) for all the experimental species. These portions for rice seedlings are shown in Figure 1 *a*. Root cultures from both these portions were initiated on MS medium containing 2.5 mg or 3 mg l^{-1} 2,4-D, in dark. For regeneration of shoots, the calli were transferred to the MS medium (devoid of 2,4-D), supplemented with 2 g l^{-1} casein hydrolysate.

Rice (cv Taipei 309) seeds placed on MS medium formed embryonic axis with distinctive shoot and root portions as shown in Figure 1 *a*. The detached root portions placed on MS + 2,4-D medium showed distinctive nodular appearance due to initiation of adventitious root primordia all through its length (Figure 1 *b*, this figure also shows formation of calli masses from the adventitious root primordia , see later part of this paper for discussion). The number of adventitious roots were noted to be higher near the root tip portions (shown by arrow in Figure 1 *b*) as compared to distal root tip portions (shown by arrowhead in Figure 1 *b*). This response of adventitious root formation was 100% as all the tested root explants formed adventitious roots (Figure 1 *b*). Further, the events of the adventitious root formation were analysed in detail. The proximal root tip portions showed a swollen appearance (possibly due to dissolution of their cortical cells) within three days of culturing (Figure 2 *a*). From the interior tissues of

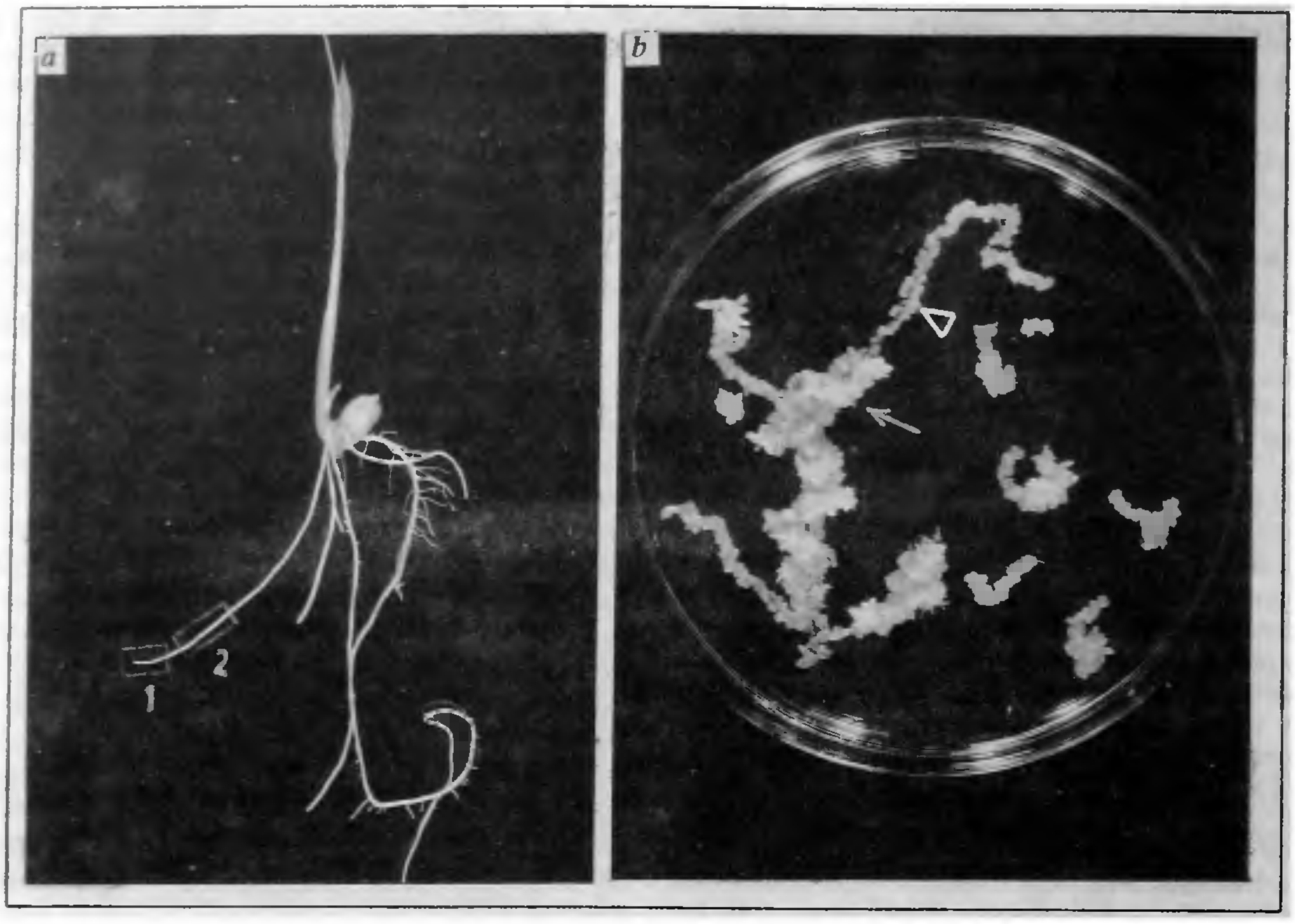

Figure 1. *a*, Seven-day-old intact rice seedling showing the growth stage of roots which were used as explants in this study. The regions of one such roots representing proximal to root tip portion (box 1) and distal to root tip portion (box 2) employed for the induction of adventitious roots are highlighted. *b*, Whole roots (after detachment from the seeds as well as the embryonic shoot portions) showing the profused adventitious roots/callusing (see text for details) after 25 days of culturing on MS medium supplemented with 2.5 mg l^{-1} 2,4-D. Arrow and arrowhead indicate proximal to root tip and distal to root tip portions of one of the roots, respectively.

the pericycle, distinctive primordia representing adventitious roots were initiated (Figure 2 *b*) which on further growth showed finger-like appearance (Figure 2 *c*). These structures showed root hairs in several instances and were positively geotropic (in later part of their development some of the adventitious roots curled backwards to touch the medium) (Figure 2 *e*). These newly-formed roots caused rupturing of the cortical cells to emerge outwards on the surface (Figure 2 *b*). The cortex was seen to slough off and degenerate. The number of adventitious roots on the surface was as many as 40 within a region of 5–6 mm of the explant from root tip portion (Figure 2 *c*). These roots appeared as rows (3–5 in number per explant) of finger (Figure 2 *c*). In subsequent course of development, callusing appeared at the basal region of the newly formed adventitious roots and proliferated towards the tip region (shown by arrow in Figure 2 *d*).

Initiation of adventitious root formation was also prominent when the portion distal to these root tips was placed on the MS + 2,4-D medium. Within 15 days of the initiation of the adventitious roots, compact and nodular callus masses were formed from the base of these roots (Figure 3 *a*). Calli derived from the explants representing distal portions were mucilaginous in the beginning; in contrast, pale yellow, compact and nodular calli obtained from proximal regions right from the beginning. The clustering of the adventitious root primordia/calli was more in explants representing proximal portions than the explants from the distal portions (as seen with the whole root section shown in Figure 1 *b*). While the precise reason(s) for this differential response are not known[1,5], it is possible that (a) proximal region has different hormonal status as compared to the distal portion, (b) the two regions have differential sensitivity to the applied 2,4-D and/or, (c) the adventitious roots appeared from preformed primordial buds which were more tightly placed at the actively dividing proximal region which got gradually spaced out in the distal part. It is thus shown in this study that the detached roots of rice respond to culturing conditions in presence of 2,4-D through the formation of incipient adventitious roots that give rise to the callus. It is noteworthy that the formation of calli from rice root explants has earlier

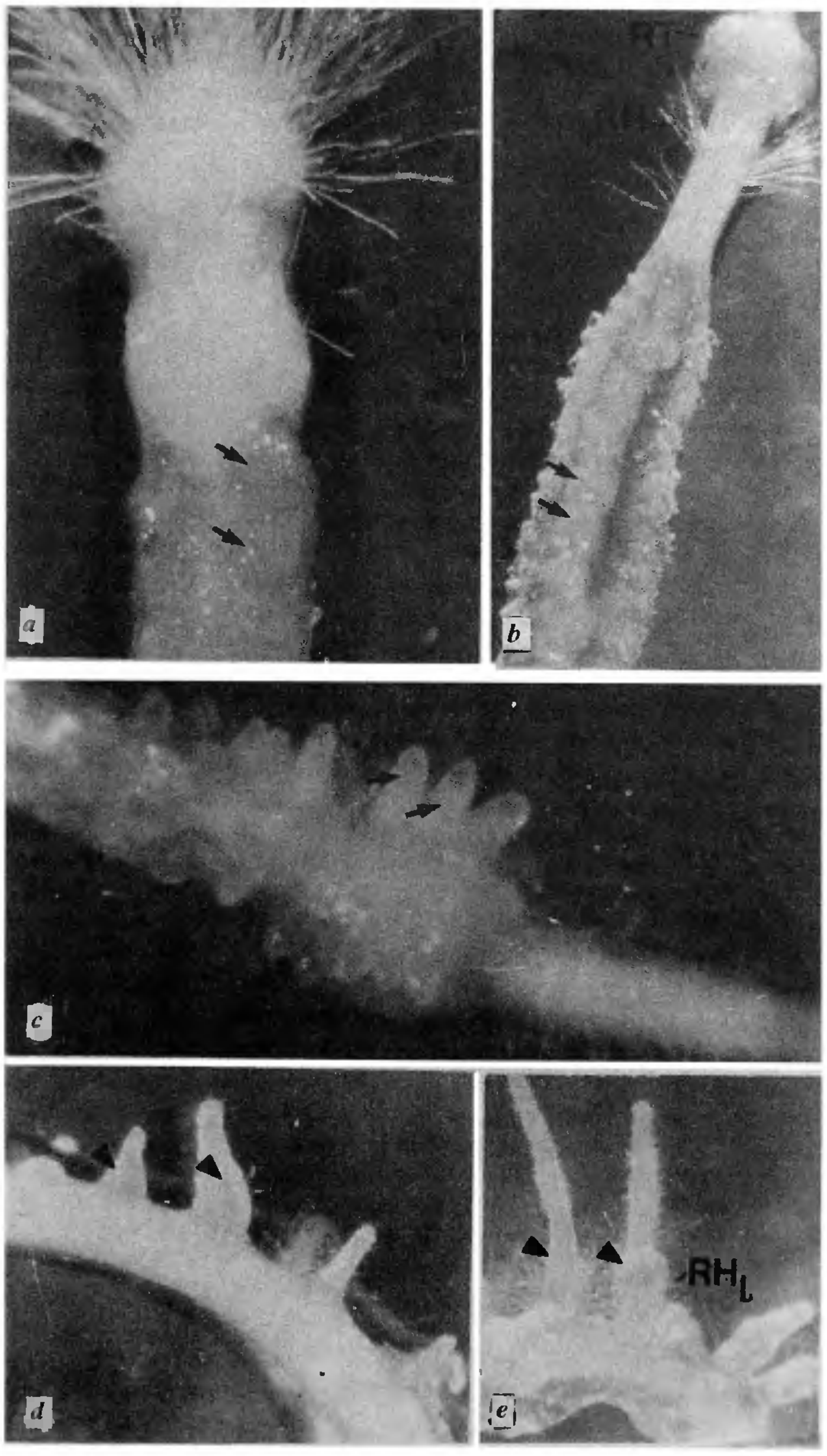

Figure 2. *a*, Appearance of the adventitious root primordia 2 days after placing of the rice root explant on the 2,4-D supplemented MS medium. Arrows indicate portions of root tip region showing early stages of the activation of adventitious primordia. *b*, The adventitious root initials (shown by arrows) appear to disrupt the cortical cells to appear on the surface. The disrupted cortex is distinctively seen here. *c*, The organized primordia of the adventitious roots appear like rows of fingers with further growth. *d*, Following a week of appearance, the basal regions of the adventitious roots show signs of swelling (shown by arrowheads). *e*, The swollen portions of the bases of the adventitious roots showing distinctive root hairs (indicated by RH_L)

been shown: however, it has been indicated that the callus[6-9] or somatic embryos[10] are formed directly from the root explants, which does not appear to be the case in our view. Importantly, callusing response in 2.5 and 3 mg l^{-1} 2,4-D supplemented media appeared similar in degree. It was further seen that the formation of adventitious roots was reduced in number when distal and proximal to root tip portions were taken from

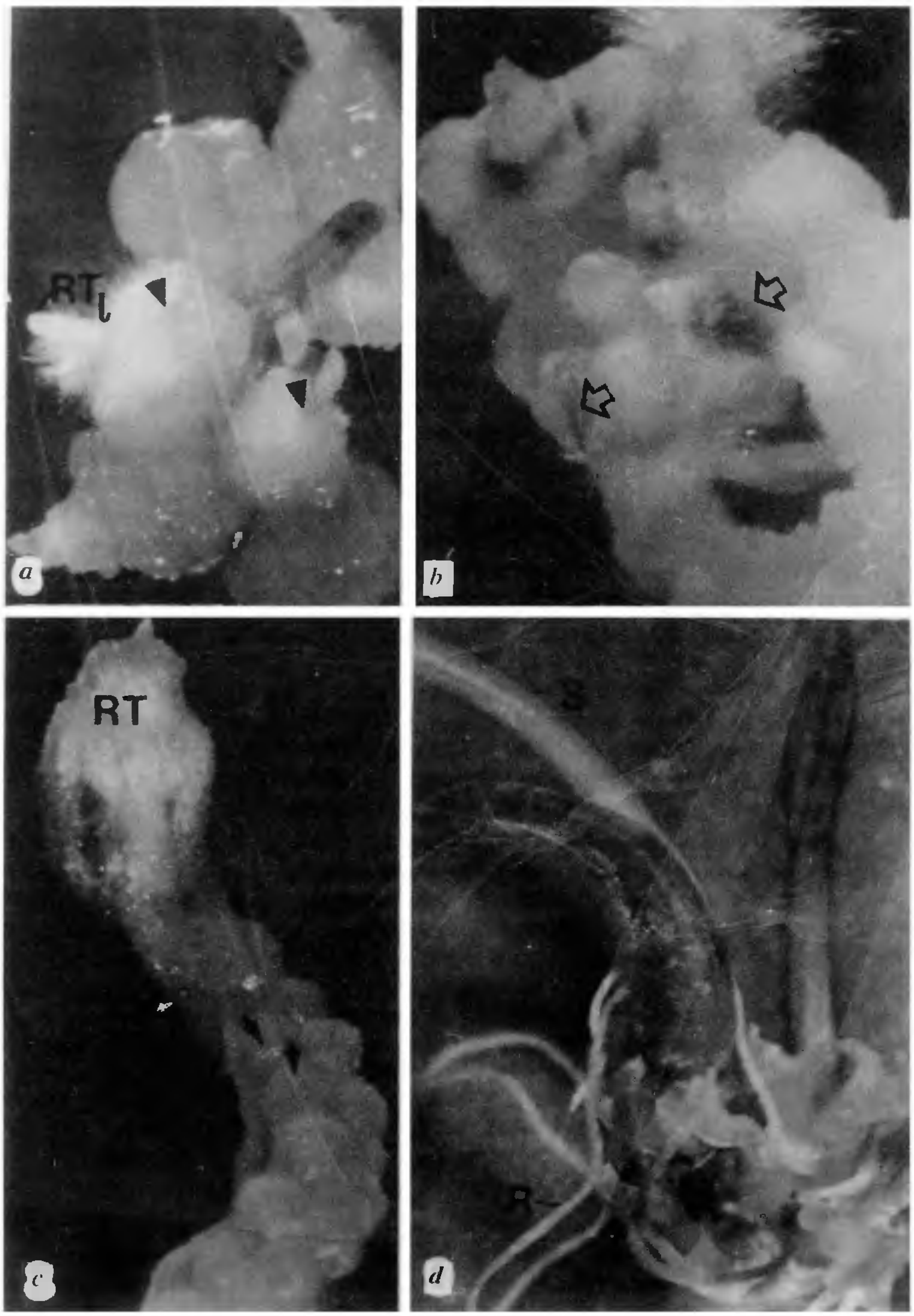

Figure 3. *a*, Appearance of the proliferated callus mass (shown by arrowhead) at the base of the adventitious roots. *b*, Formation of shoot primordia on transfer of the calli to hormone-free MS medium (indicated by the empty arrows). *c*, Appearance of the adventitious root primordia (shown by arrows) in cultured root explants in wheat. The tip portion of the root explant (indicated by RT) is shown. *d*, Regenerated rice plantlet from the callus formed from the basal portions of the adventitious root.

10-day-old rice seedlings, as compared to 7-day-old seedlings mentioned above. The adventitious roots were not formed when roots from 14-day-old seedlings were tested. These observations indicated that formation of adventitious roots is an age-dependent response. Similar findings have earlier been documented for other plant species[1].

Formation of adventitious roots and their subsequent callusing from the basal regions in response to culturing conditions as shown above for rice cv Taipei 309 was seen in three other cultivars of rice as well (IR 54, Pusa 169 and Basmati 370). The degree of the response too was comparable in all the four cultivars. Apart from rice, wheat (Figure 3 *c*) and maize roots also showed similar responses (both in terms of adventitious root formation and callusing). These observations indicate that the culturing conditions employed here (i.e. MS medium + 2.5 mg l^{-1} 2,4-D; aseptic milieu) are highly suitable for the formation of adventitious roots from the roots of the above seedlings. In case of brassica, adventitious root formation was appreciably delayed and the rooting and callusing response was much less conspicuous as seen in rice. This might be a reflection of the requirements of different culturing conditions (in terms of 2,4-D concentrations, etc.) for the brassica seedlings which remains to be analysed. Callus pieces obtained from both explants (representing proximal and distal portions) of rice (Taipei 309) showed formation of a large number of green spots when transferred to the regeneration medium (Figure 3 *b*). The proximal root tip-derived calli appeared more efficient in producing green shoot initiation spots than the distal root-derived calli. Importantly, the proximal explants showed significant frequency of forming green spots (88%) even when placed in the regeneration medium after 9 days of callus initiation. The formation of green spots was 75% even after 36 days on the callus initiation medium. The formation of green shoot regenerated from such green spots is shown in Figure 3 *d*.

Techniques for genetic transformation of rice such as electroporation , use of particle gun and more recently, through *Agrobacterium* have been optimized to a great extent[11]. The availability of embryogenic calli in large amounts is an important input in rice biotechnology research[12]. Methods which can increase root mass are desirable for the experiments involving root explants for raising calli. The suitability of the calli derived from the adventitious roots as compared to, for example, the seed-derived calli depends on the capacity of these calli to regenerate plantlets. Regeneration of shoot primordia in calli formed from adventitious roots was found to be high (both in terms of number of shoot primordia formed per callus as well as proportion of calli which formed the shoot primordia) in this study. It has earlier been shown that plantlets regenerated from the root calli do not show any morphological abnormalities and are fertile[10]. From this, it is inferred that the callus from adventitious roots is embryogenic. The cells taken from these calli were isodiametric, rich in cytoplasmic inclusions and were scantily vacuolated, indicating their high meristematic activity (data not shown).

In conclusion, we highlight the following two points. Firstly, the formation of adventitious root primordia was seen to be a highly-synchronized response elicited by application of auxin. It represented a case of hormone-mediated redifferentiation (formation of adventitious roots) and redifferentiation (formation of calli from the base of the adventitious roots). It should be rewarding to use this system to work out early gene expression changes in studies aimed at understanding the basics of morphogenetic events under the control of hormonal action. Secondly, it is important to appreciate that the quantity of primary callus which can be obtained from root explants is significant as 4–5 roots are formed from each seed (up to 7 days of growth till which time the response of adventitious root formation was noted to be high in this study, see Figure 1 *a*) and each of these roots can give a large number of primary calli (more than 100 from a single root, see Figure 1 *b*). It is left to be determined how competent the adventitious root-derived calli are in uptake and integration of the foreign genes as compared to calli obtained from other explants (such as seeds). We are presently investigating utility of root-derived calli in genetic transformation experiments.

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ACKNOWLEDGEMENTS. Financial assistance to our laboratory from the Rockefeller Foundation, USA is gratefully acknowledged. D.M. thanks University Grants Commission, New Delhi for the award of Senior Research Fellowship. We thank Dr M. T. Jackson, International Rice Research Institute for the supply of IR 54 and Taipei 309 rice seeds. We also thank Heads of Genetics Division, Cereal Laboratory and NRC on Biotechnology (IARI) for the seeds of Basmati 370 and Pusa 169 rice cultivars, maize and brassica, respectively.

Received 16 May 1997; accepted 16 July 1997