

Plant regeneration from mesophyll protoplasts of *Rorippa indica* (L.) Hiern, a wild crucifer

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In *Rorippa indica*, a wild crucifer, high-frequency plant regeneration through organogenesis (~38%) was achieved from *in vitro*-grown leaf explants. When the same-aged *in vitro*-grown leaves were utilized for protoplast isolation, $\sim 4 \times 10^6$ protoplasts g^{-1} leaf tissue were obtained after digestion with an enzyme solution containing cellulase and macerozyme. A maximum of ~66% first division, ~53% second division and ~35% plating efficiency were obtained when protoplasts were plated at 5×10^4 protoplasts mL^{-1} plating density. All the 528 protoplast-derived calli tested for regeneration exhibited shoot organogenesis. Regenerated shoots were transferred to soil after rooting and hardening. Chromosome analysis of microspore mother cells from pot-grown protoplast regenerated plants showed that all were diploid ($2n = 32$).

RORIPPA indica (L.) Hiern, a wild crucifer, is found in the plains of West Bengal, India in a rosette form in winter, and thereafter, it bolts and develops as highly branched bushy habit in the summer. *R. indica* has a large number of pods and seeds per pod. It is aphid-tolerant and its seeds contain a high percentage of oleic acid (26.65%) and linoleic acid (26.94%), and a moderate percentage of erucic acid (24.40%). As sexual crossing fails between *R. indica* and oil-yielding *Brassica* species (500 crosses each were tried with *B. juncea* and *B. campestris*, unpublished), protoplast fusion might be an alternative way to introgress desirable characters of *R. indica* to oil-yielding *Brassica* species. Plant-regeneration protocol from the parental protoplasts sometimes becomes helpful to regenerate the somatic hybrid. Successful regeneration from protoplasts of different *Brassica* species have been reported^{1,2}, but not from *R. indica*. The present communication describes high-frequency plant regeneration from leaf explants as well as from mesophyll protoplasts of *R. indica*.

Seeds of *R. indica* (L.) Hiern were collected from Kolkata. Seeds were surface-sterilized in 0.2% HgCl_2 for 6 min followed by 3 rinses with sterile water. They were germinated on plant growth regulator-free MS³ medium (MS-H) in the dark at $25 \pm 1^\circ\text{C}$. The apical portion of one-month-old seedlings was cut and cultured on MS-H medium. The plants were multiplied under *in vitro* condition at $25 \pm 1^\circ\text{C}$ with 16/8 h light/dark photoperiod (light intensity was maintained at $24 \mu\text{Em}^{-2} \text{s}^{-1}$ of PAR irradi-

ance of white fluorescent tubes) by transferring apical shoot tips and nodal segments to fresh MS-H medium once a month. To study the suitability of leaf explants for plant regeneration, multiple incisions (ca 2 mm apart) were made on the lower surface of the leaves taken from 15-day-old *in vitro*-grown plants and placed on MS medium supplemented with various combinations of plant growth regulators (PGRs), NAA (0.27–5.37 μM), BAP (2.22–13.32 μM), IAA (0.29–28.54 μM), kinetin (0.23–13.94 μM), GA_3 (0.14–2.87 μM) and 0.8% agar, keeping the abaxial side upward. The cultures were incubated under 16/8 h light/dark photoperiod at $25 \pm 1^\circ\text{C}$ for 30 days. For protoplast isolation, 1 g chopped leaves (taken from 15-day-old *in vitro*-grown plants), pre-incubated 30 min in the osmoticum solution (0.6 M mannitol + 0.0136 M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, pH 5.8) at $25 \pm 1^\circ\text{C}$ was digested in 10–20 ml enzyme mixtures containing cellulase Onozuka R-10 (0.25–1.0%) and macerozyme R-10 (0.25–1.0%) in osmoticum solution, pH 5.8 at $25 \pm 1^\circ\text{C}$ for 11–14 h in the dark and with slow shaking (50 rpm). Once the protoplasts were formed, the enzyme solution was decanted after centrifugation for 3 min at 100 g. The protoplast pellet was suspended in 1 ml osmoticum solution and poured over 10 ml of 20% (w/v) sucrose solution in a 15 ml centrifuge tube. After 10 min centrifugation at 100 g, the protoplast layer was collected. The purified protoplasts were then washed thrice with osmoticum solution through centrifugation at 100 g for 3 min each and resuspended in osmoticum solution. Fluorescein diacetate-tested⁴ protoplasts were plated in different media (2 ml per 35 mm \varnothing plastic petri dishes at a density of $3\text{--}8 \times 10^4$ protoplasts mL^{-1}) containing MS or MK₃ (ref. 5) or 8P⁶ basal medium supplemented with various combinations of PGRs, 2,4-D (0.23–4.52 μM), NAA (0.27–5.37 μM), BAP (0.44–13.32 μM), IAA (0.29–28.54 μM), kinetin (0.46–13.94 μM), GA_3 (0.14–2.87 μM) and MES (0–5 mM). For the first 14 days the culture plates were kept in the dark and thereafter exposed to light under a 16/8 h light/dark photoperiod at $25 \pm 1^\circ\text{C}$. The liquid medium with specific PGR combinations was changed every seven days. On the 28th day, the liquid medium was replaced by a semi-solid medium. Protoplast-derived macro calli were transferred to LR1 medium (Table 1) on day 38 for shoot regeneration. The regenerated shoots were then isolated and cultured in MS-H medium for further growth and root induction. The rooted plants were transferred to the pots containing a mixture of loam soil and cow-dung manure (2:1), watered and covered with transparent polypropylene bag to maintain high humid condition. The pots were kept in daylight for seven days after which the polypropylene cover was removed and the potted plants were maintained in the open environment. Flower buds were collected from the pot-grown plants at 10 a.m., anthers were removed and smeared on glass slides and stained with 1% acetic-carmin. The stained chromosomes at meiotic metaphase-I were obser-

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ved under the compound microscope and photographed. The compositions of culture media used were as follows: for all the cultures MS basal medium and 5 mM MES (except in LR1 medium) were used and pH was adjusted to 5.8. RA₁, see Table 2; RA₂, 0.45 μ M 2,4-D + 0.54 μ M NAA + 8.88 μ M BAP + 0.29 μ M GA₃ + 0.4 M glucose + 0.0292 M sucrose; RA₃, 0.54 μ M NAA + 8.88 μ M BAP + 0.29 μ M GA₃ + 0.2 M glucose + 0.0584 M sucrose; RA₄,

0.54 μ M NAA + 8.88 μ M BAP + 0.29 μ M GA₃ + 0.0876 M sucrose + 0.6% agar; LR1, see Table 1. Student's *t* test was employed to test the significance between treatments.

Among the five most responsive media, the maximum (37.62 ± 5.26) and the minimum (6.96 ± 2.31) percentage shoot regeneration (Figure 1) through organogenesis per leaf explant was obtained on LR1 and LR4 medium, respectively between 14 and 21 days (Table 1). The number of shoots produced on LR1 medium was significantly different from that of LR3, LR4 and LR5 media, but was not significantly different from the number produced on LR2 at $P \leq 0.05$. Out of 1329 plants regenerated, 100 rooted plants were transferred to the field. Meiotic chromosome analysis of 35 plants revealed that all were diploid ($2n = 32$).

A maximum of 4×10^6 protoplasts (30–50 μ m \varnothing) (Figure 2a) were obtained per gram of leaf when digested in 10 ml of the enzyme mixture containing 0.25% cellulase and 0.5% macerozyme for 12 h. Among 98% viable protoplast-derived cells, $66.28 \pm 9.62\%$ and $52.56 \pm 10.31\%$ underwent 1st and 2nd division (Figure 2b), respectively within 6–8 days in RA₁ medium (Table 2), at a plating density of 5×10^4 protoplasts ml⁻¹. Five out of the 21 different media tested gave a good division response. The number of cells undergoing 1st division on RA₁ medium was significantly different from the cells undergoing 1st division on RC₁ and RE₁ media, but was not significantly different from the cells undergoing 1st division on RB₁ and RD₁ media at $P \leq 0.05$. The difference in 2nd division of RA₁ medium with RB₁, RC₁ and RE₁ media were significant, but not with RD₁ medium at $P \leq 0.05$. After protoplast plating, 8–16 cell stages (Figure 2c) were



Figure 1. Shoot regeneration from leaf explants of *Rorippa indica* on LR1 medium. Bar = 1.2 cm.

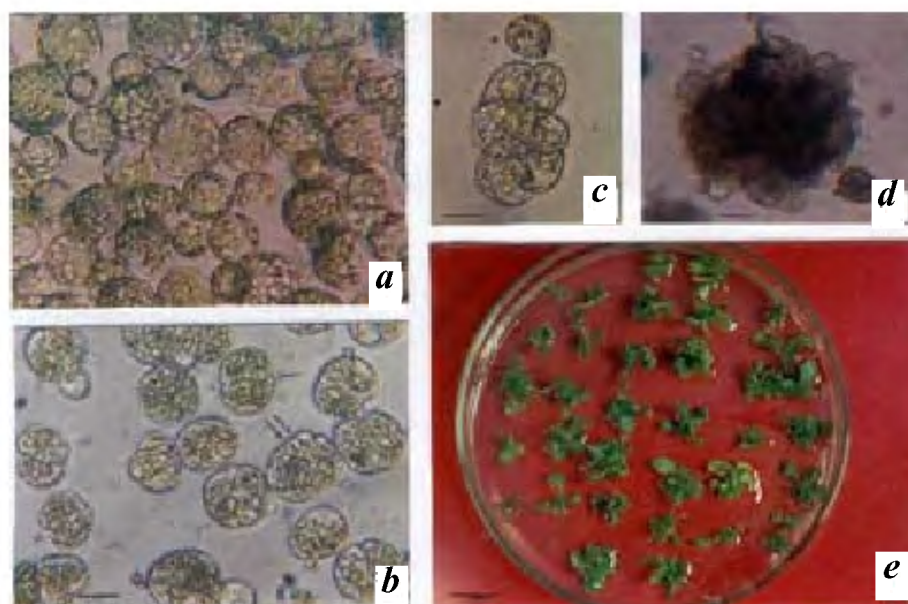


Figure 2. Different stages of cells derived from protoplasts of *R. indica* leading to plant development. (a) Freshly isolated protoplasts, (b) first (\uparrow) and second ($\uparrow\uparrow$) divisions of cells derived from protoplasts, (c) eight-celled stage, (d) micro-colony and (e) shoots developed from calli derived from protoplasts. Bar = 25 μ m for a–d and 1.2 cm for e.

Table 1. Number of shoots regenerated per leaf explant of *Rorippa indica* on MS basal medium supplemented with five different combinations of plant growth regulators (in each medium 0.0876 M sucrose and 0.8% agar were used and pH was adjusted to 5.8)

| Medium code | Plant growth regulator (μM) | | | | | No. of shoots/ leaf explant ^a |
|-------------|--|-------|-------|---------|-----------------|--|
| | NAA | IAA | BAP | Kinetin | GA ₃ | |
| LR1 | 0.54 | — | 8.88 | — | 0.29 | 37.62 \pm 5.26 |
| LR2 | 1.07 | — | 8.88 | — | 0.29 | 26.85 \pm 4.49 |
| LR3 | — | — | 13.32 | — | 0.29 | 15.18 \pm 3.63 |
| LR4 | — | 14.27 | — | 6.97 | 0.29 | 6.96 \pm 2.31 |
| LR5 | — | 28.54 | — | 6.97 | 0.29 | 10.75 \pm 2.66 |

^aEach value shows the mean of three replications (20 leaves in each replication) \pm SE of the mean.

Table 2. Per cent of cells obtained from mesophyll protoplasts of *R. indica* showing first and second division in the five most responsive media. Each liquid medium contained MS basal supplemented with 0.6 M glucose and 0.29 μM GA₃. Plating density was maintained at 5×10^4 protoplasts ml^{-1}

| Medium code | Plant growth regulator (μM) | | | | | Percentage of cell dividing ^a | |
|-----------------|--|------|------|-------|---------|--|-------------------|
| | 2,4-D | NAA | BAP | IAA | Kinetin | 1st division | 2nd division |
| RA ₁ | 0.90 | 1.07 | 8.88 | — | — | 66.28 \pm 9.62 | 52.56 \pm 10.31 |
| RB ₁ | 1.13 | 5.37 | 4.44 | — | — | 55.14 \pm 9.27 | 38.21 \pm 7.62 |
| RC ₁ | 2.26 | 5.37 | 4.44 | — | — | 47.89 \pm 6.41 | 31.57 \pm 5.83 |
| RD ₁ | — | — | — | 11.42 | 13.94 | 56.71 \pm 7.26 | 45.73 \pm 6.28 |
| RE ₁ | — | — | — | 28.54 | 13.94 | 53.25 \pm 8.52 | 30.29 \pm 7.13 |

^aEach value shows the mean of three replications (five observations in each replication) \pm SE of the mean.



Figure 3. Pot-grown plant of *R. indica* derived from protoplast. (Inset) Metaphase I chromosomes of a pollen mother cell of the same plant showing 16 bivalents. Bar = 5 cm.

observed between 13 and 18 days. The maximum plating efficiency of $35.14 \pm 8.38\%$ was obtained in RA₂ me-

dium after 20 days following 14 days culture in RA₁ medium. On day 21, a third medium change was carried out with RA₃ diluting the cell density to one-fourth concentration. This resulted in micro-colony formation (Figure 2d) within 21–28 days. On day 28, a fourth medium change was carried out with RA₄ medium and macrocolonies were formed within 7–10 days. From 528 protoplast-derived calli, a total of 7492 shoots were regenerated through organogenesis. The calli started greening after 6 days and shoot regeneration (Figure 2e) started from day 8 and peaked within 12 days. Chromosome counts were carried out from pollen mother cells in 35 out of 100 field-transferred plants. All were found to be diploid ($2n = 32$) (Figure 3) and flowered and fruited normally. Starting from protoplast plating, 50–60 days were needed for shoot organogenesis. Thirty-five per cent plating efficiency of protoplasts and 100% plant regeneration from calli obtained from protoplasts with 14.18 ± 2.21 shoots per callus were achieved in the present study. The 100% plant regeneration from protoplasts of Cruciferae reported so far, is only from mesophyll protoplasts of *Isatis indigotica*, by Hu *et al.*⁷. In cruciferous protoplast culture, inhibition of growth due to browning of culture as a result of formation of phenolic compounds was observed^{8–11}. Addition of MES and change of culture media at frequent intervals helped overcome this problem

in *R. indica* protoplast culture. Maintenance of high osmotic condition (0.6 M glucose) up to the second week of culture is necessary for the stability of the protoplasts. Gradual lowering of the osmoticum by lowering of glucose, increasing sucrose, and a gradual lowering of auxin (both 2,4-D and NAA in the RA₂ medium and finally elimination of 2,4-D in RA₃, RA₄ and LR1 media) are important for high plating efficiency of protoplasts and high-frequency of plant regeneration from calli of protoplast origin in *R. indica*.

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Molecular diversity in *Phyllanthus amarus* assessed through RAPD analysis

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A collection of *Phyllanthus amarus* was made from various parts of India to determine the extent of genetic variability using analysis at DNA level. RAPD profiling of 33 collections from different locations, covering states of Tamil Nadu, Karnataka, Maharashtra, Gujarat, Assam, West Bengal, Tripura, Uttar Pradesh, Punjab and Haryana was generated. Analysis through UPGMA revealed up to 65% variation among these accessions. However, intra-population variation was found to be much larger in the accession from the southern part of the country. Nevertheless, inter-population variation also overlaps in the phylogenetic clustering, which is understandable from the natural dissemination of this plant species as a weed that has spread across the geographical boundaries. The study indicates the random hybridization across the populations falling within the range of possible cross-pollination in terms of physical distance. A study of these accessions at a single location, allowing free mating, would throw light on the extent of cross-pollination and genetic flow.

THE genus *Phyllanthus* L. of the family Euphorbiaceae consists of about 800 species, of which 200 are American, 100 African, 70 from Madagascar and the remaining Asian and Australasian¹. *Phyllanthus amarus* is an important medicinal plant species due to its antiviral properties, useful against hepatitis infection. The species is also used in stomach ailments like dyspepsia, colic, diarrhoea, dysentery, dropsy, urinogenital problems and also as external application for oedematous swelling and inflammation. This is an important ingredient in many ayurvedic preparations, especially for the treatment of jaundice. The taxonomic revision on this genus by Webster included closely-related genera *P. amarus*, under the sub-section *Swartziani* of the section *Phyllanthus*. The nomenclature, taxonomic distinctness and close relatives of *P. amarus* were addressed in detail based on morphology and geographical distribution^{2–4}. It is said to be related to *P. abnormis*, which is endemic to sandy areas in Texas and Florida of southern USA². It is therefore most likely that *P. amarus* originated in the Caribbean area as a vicarious species of *P. abnormis* of the southern United

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