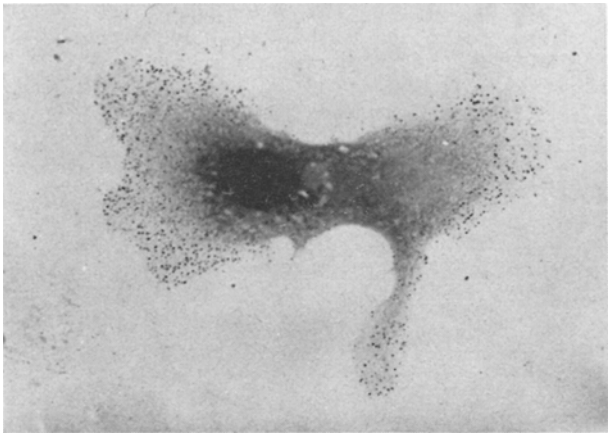
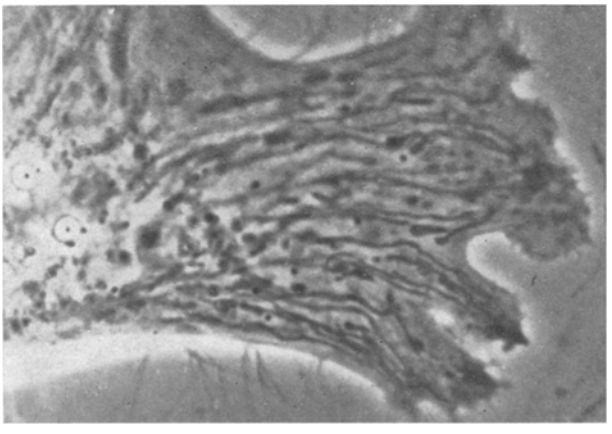
a



b



a Cytoplasmic radioactivity after incubation with tritiated thymidine (cytoplasmic DNA synthesis) in a cell from human ascitic fluid, cultivated in vitro. Giemsa, × 1200.
b Cytoplasmic expansion of a living cell from human ascitic fluid, cultivated in vitro. × 3500.

38 °C. Afterwards they were washed 3 times with calcium- and magnesium-free phosphate buffered saline (CMF-PBS), then fixed 10 min with ethanol 95%:acetic acid (9:3) and finally air dried.

For histoautoradiography we used the technique described by Gahan[2]: Kodak Nuclear Track Emulsion, type NTB 2:distillated water, 1:1; developer Kodak D 19; duration of exposure: 10 days at 4 °C. 2 controls were used for the activity of DNA synthesis: first neutral deoxyribonuclease (3200 U/mg; 0.05 mg/ml, 24 h digestion) on the fixed cells; secon chick embryo fibroblasts cultivated for 3 days and treated with the same histoautoradiographic technique as the cells of the ascitic fluid.

The cells of human ascitic fluid, cultivated in vitro, showed an activity of cytoplasmic DNA synthesis which was at times very important (figure, a). This activity was present in 5–10% of cells, it was not present in the cytoplasm of fibroblasts. They showed an activity of DNA synthesis which was exclusively nuclear. After DNAse treatment this activity disappeared. The activity of cytoplasmic DNA synthesis in the cells of human ascitic fluid is apparently not concomitant with a nuclear DNA synthesis; this affirmation is supported by the fact that we did not see any labelled nucleus in the same cells. We note here that the cells showing an activity of cytoplasmic DNA synthesis do not divide by mitosis during the contact with the thymidine-^{3}H.

These cells exhibited large cytoplasmic expansions, characterized by a very rich network of mitochondria (figure, b). Cytoplasmic mitochondrial DNA synthesis and its part in cell metabolism was recently discussed by Ried[3], Chèvremont[4] and Mitchison[5] and it does not seem to be elucidated.

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Isolation and fusion studies on protoplasts from pollen tetrads

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Summary. Different enzymes were tested for isolation of intact protoplasts from pollen tetrads. About 80% isolation was achieved from pollen tetrads of Cajanus cajan and Zea mays and about 60% from Luffa cylindrica and Lycopersicon esculentum after 4 h of treatment with 5% cellulase. When these mononucleate protoplasts were incubated in presence of 0.05 M $CaCl_2$ in 0.3 M glucose at pH 10.5, 70–80% fusion was achieved. Fusion was rare in sodium nitrate solutions.

Apart from using haploid pollen tetrad protoplasts as a starting material for the production of haploid plants[3], they are also suitable for somatic genetics studies, since mutants would easily be detectable in haploids[4,5]. Fusion experiments with haploid protoplasts could be useful for the study of nuclear behaviour in the fused cell and also for the regeneration phenomenon. Till now most of the studies concerning protoplast isolation, fusion and culture have been restricted to mesophyll and callus protoplasts[6–11]. The present study deals with the feasibility of isolating intact protoplasts from pollen tetrads from various plant species and their response to different physical and chemical agents to cause fusion.

Materials and methods. Pollen tetrads and pollen grains from Cajanus cajan, Zea mays, Luffa cylindrica and Lycopersicon esculentum grown in outdoor conditions have been tested for isolation of intact protoplasts. The actual developmental stages were assessed by examining one of the anther of a flower or floret under microscope, and the rest were sterilized with 5% calcium hypochlorite for 5 min and were then transferred to the enzyme solution (0.2–0.4 ml per anther) containing 0.45 M mannitol at pH 5.4. The anthers were cut with a sharp razor to squeeze out the tetrads or pollen grains with gentle pressing and the remaining debris was removed. Incubation of the released pollen tetrads and pollen grains have

Effect of enzyme-osmoticum on release of protoplasts from pollen tetrads of Cajanus cajan

Treatment	Period of incubation (h)		
	2	4	6
Pectinase 5%	–	–	–
Driselase 5%	–	+	+
Cellulase 5%	–	+++	+++
Pectinase 5% + driselase 5%	–	+	+
Pectinase 5% + cellulase 5%	–	++	+++
Driselase 5% + cellulase 5%	–	+++	+++

–, 0%; +, 40–50%; ++, 50–60%; +++, 70–80%.

been carried out in humid chambres at 28 °C. 3 enzymes namely, pectinase (Serva), driselase (Kyowa Kakko Co., Othemachi Tokyo) and cellulase 'Onozuka SS' (All Japan Biochemicals), have been tested alone or in combination as shown in the table. Enzyme solutions were previously sterilised by filtration through a Millipore filter (0.45 μm). All such operations were carried out in aseptic conditions. For the induction of fusion through temperature treatments, isolated protoplasts have been suspended in SH medium[12] and have then been kept in refrigerator at 4 °C or kept in incubators at 45 °C. For chemical inducation of fusion, 0.45 M mannitol, 0.56 M sucrose, 1 M $NaNO_3$, 0.4 M $NaNO_3$ and Keller and Melchers's[13] high pH and high calcium solution with slight modification (buffer: 45.8 ml 1 N NaOH and 54.2 ml 0.1 M glycine- 0.1 M NaCl; to make it 0.3 M glucose and 0.05 M $CaCl_2$ at pH 10.5) have been tested. After isolation, the protoplasts have been washed with 0.45 M mannitol with low speed centrifugation (80 × g) and have then been suspended in the fusion solution to be tested. They were kept in suspension for 4 h. Samples were taken at 10 min interval for study of agglutination and fusion. When sufficient agglutination and fusion had been observed, they were washed with 0.45 M mannitol and were resuspended in SH medium[12] for further study.

Results and discussions. Effect of 3 different enzymes alone, or in combinations on releasing protoplasts from pollen tetrads of Cajanus cajan, is shown in the table. Cellulase at 5% level was found to be most effective in releasing intact protoplasts in all the 4 experimental systems examined. Pectinase was found to be ineffective as was reported earlier[14], and driselase was less effective than cellulase. It was also observed that driselase in combination with cellulase does not acelerate or increase the rate of isolation. It took about 4 h to release 50–60%

1 This work was supported by a grant from the Indian Council of Agricultural Research, New Delhi (No. 1-32/73, Ed. I) in the form of a Senior Fellowship awarded to P. C. D.

2 Work carried out at Molecular Cytogenetics Research Unit, Dept. of Genetics and Plant Breeding, Banaras Hindu University, Varanasi, India.

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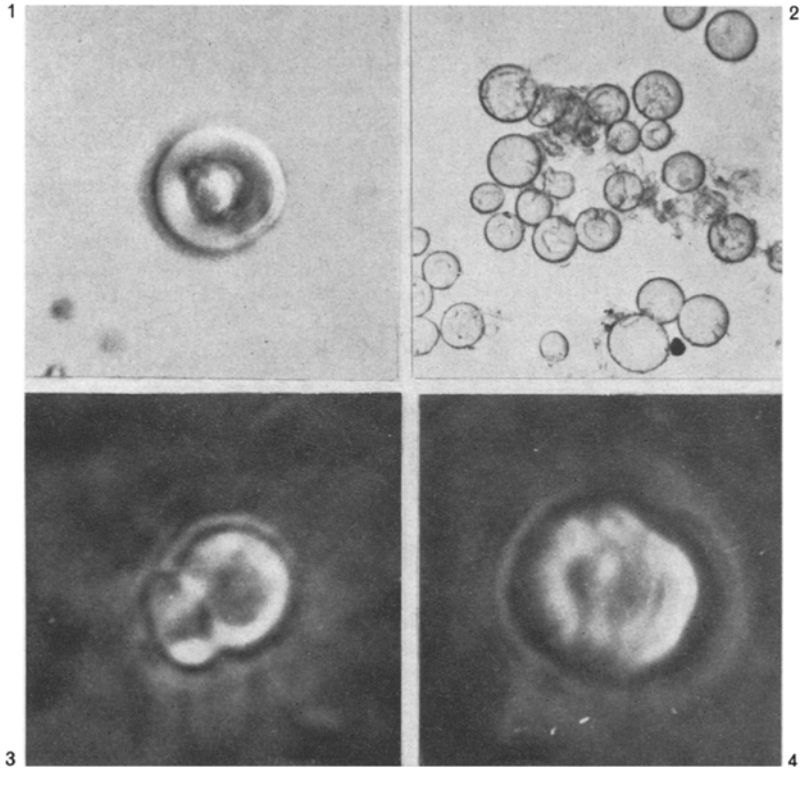

Fig. 1. Microspore protoplast of Cajanus cajan having a large nucleus. Phase contrast. × 690.

Fig. 2. Isolated microspore protoplasts of Zea mays showing adherence after 10 min of treatment with high pH and high calcium solution. × 420.

Fig. 3. 2 microspore protoplasts of Cajanus cajan in the process of fusion. Phase contrast. × 690.

Fig. 4. Fused microspore protoplasts of Cajanus cajan gradually becoming round with mixing of the cytoplasm. Phase contrast. × 750.

protoplasts from pollen tetrads of Luffa cylindrica and Lycopersicon esculentum when treated with 5% cellulase, whereas within the same period about 70–80% isolated protoplasts was obtained in the case of Cajanus cajan and Zea mays. Young pollen tetrads were found to be most responsive to the enzyme treatment, with resulting release of protoplasts. Spherical, non-vacuolate protoplasts thus isolated were fairly uniform in size, having a large nucleus at the centre (figure 1). Pollen grains did not yield release of protoplasts, probably because of their exines being not responsive to either of the enzymes tested.

During the isolation process, less than 3% spontaneous fusion was observed. Even after following the method developed by Ito and Media[15] to cause spontaneous fusion, we did not observe more than 5% fusion. Agglutination or fusion was not induced through high or low temperature treatments.

Neither 0.45 M mannitol nor 0.56 M sucrose could induce fusion even after 4 h of treatment. But treatment with 0.4 M sodium nitrate for 4 h induced about 8–10% fusion. Over plasmolysis resulted when 1 M sodium nitrate was used. However, the most significant result was observed through treatment with Keller and Melchers's[13] high pH and high calcium fusion solution with the modification. Adherence of the naked protoplasts could be seen even after 10 min of treatment (figure 2). The rate of agglutination and fusion increases with the increase of time and after 30 min of treatment about 70–80% of agglutination was observed. Fusion of the adhered cells proceeds quickly to a dumb-bell shape structure (figure 3) followed by formation of a spherical shape (figure 4) with the mixing of the cytoplasm. In about 5% cases, nuclear fusion was observed after about 10 h of culturing. When several protoplasts were seen to fuse together, multilobed structures were evident, which after a lapse of time rounded off.

Work is in progress to find appropriate cultural conditions necessary for these haploid protoplasts for carrying out elaborate somatic genetics studies.

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Interspecific protoplast fusion and complementation in *Aspergilli*

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Summary. Protoplast fusion and nutritional complementation between auxotrophic mutants of *Aspergillus nidulans* and *Aspergillus fumigatus* has been achieved. It is concluded that the nutritional complementation may be due to interspecific aneuploidy.

We previously reported[2] that high-frequency intraspecific protoplast fusion and heterokaryon formation were obtained in *Aspergilli* and *Penicillia*. We now describe successful experiments to fuse protoplasts of auxotrophic mutants of taxonomically distant *A. nidulans* and *A. fumigatus*, resulting in nutritional complementation and interspecific aneuploidy of hyper-haploid type.

Material and methods. Stable mutants requiring lysine (lys) and adenine (ade) were produced by UV-irradiation from *A. nidulans* R21 (yellow conidia, and requiring *p*-aminobenzoic acid)[3,1] and *A. fumigatus* 5085 (wild-type)[1]. Back-mutation has never been observed with these mutants. The methods of protoplasts formation, fusion with polyethylene glycol (PEG) and regeneration were carried out under optimal conditions[2]. The complemented colonies were selected on minimal medium containing *p*-aminobenzoic acid (PABA). The complementation frequency is expressed as the number of colonies developing after PEG treatment in minimal medium compared to the number growing in yeast-extract medium. The method of staining conidial nuclei was based on that of PUHALLA[4].

Result and discussion. In PABA-containing minimal medium protoplasts were able to regenerate and develop into colonies in low frequency after interspecific protoplast fusion had been induced between *A. nidulans* lys and *A. fumigatus* ade or *A. nidulans* ade and *A. fumigatus* lys. We shall deal here merely with cases when only *A. nidulans* could be recovered from the interspecific fusion products; opposite cases, when only *A. fumigatus* could be regained, are also known and will be reported elsewhere. The main characteristics of these interspecific products are as follows.

In interspecific protoplast fusion, the complementation frequency was of the order of 10^{-5} whereas in intraspecific fusion as high as 40 to 60% can regularly be attained[2,5]. Complementation was never observed when mycelia of the two species were mixed and incubated in an attempt to achieve hyphal fusion, whereas intraspecifically complementation is common with these mutants and with others[6–9].

The colonies resulting from interspecific fusion were thick, irregularly growing and differed markedly in appearance from intraspecific ones (Figure 1). The hyphae often exhibited deformations (Figure 2). Conidium-formation was infrequent, with one nucleus in each conidium. Diploidization has never been found. Nevertheless, conidia from interspecific colonies were able to germinate

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