DNA, 25 μg of random primers, 100 mM Tris-Cl (pH 8.0), 8 mM $MgCl_2$, 1% 2-mercaptoethanol, 1 mM each of dATP, dCTP, dTTP, 20μCi of 5'-(α-^{32}P)dGTP and 5 units of AMV reverse transcriptase. The reaction mixture was incubated at 42 C for 1.5 h. The incorporation of label was checked by electrophoresis of the labelled DNA in 1.2% agarose gel followed by autoradiography. At least three additional forms of DNA were efficiently labelled in the diseased sample (DL-DNA) as compared to its healthy counterpart (HL- DNA) which only showed the labelling of a high molecular weight DNA (Figure 1,*a*, lanes 1 and 2).

Unlabelled HL-DNA and DL-DNA electrophoresed in 1.2% agarose gel and transferred on nitrocellulose membrane were probed with the labelled mixture prepared from total DL-DNA. No band was observed in HL-DNA lane (Figure 1,*b*, lane 1) while in the DL-DNA lane six different bands marked 1–6 on right side (Figure 1, *b*, lane 2) were apparent. These bands, by analogy to DNA forms present in tomato golden mosaic virus-infected tissue are the various forms of ds- and ss-DNA present in *Acalypha* leaves affected by the virus[7]. The absence of high molecular weight band in healthy tissue may be due to its inefficient transfer to the nitrocellulose membrane.

The approach has worked well in the case of two other geminiviruses affecting mung bean and tomato during our recent observation. The strategy would help quick identification of different topological forms of viral DNA and their transcripts present in the infected tissue. It will also be useful in differentiation of other geminiviruses occurring in tropical countries without going through the difficult process of virus purification.

1. Goodman, R. M., in *Handbook of Plant Virus Infections and Comparative Diagnosis* (ed. Kurstak, E.), Elsevier, Amsterdam, 1981, p. 879.
2. Srivastava, K. M., Raizada, R. K. and Singh, B. P. in *Perspectives in Plant Virology* (eds. Gupta, B. M., Singh, B. P., Verma, H. N. and Srivastava, K. M.), Print House, Lucknow, 1985, p. 153.
3. Bock, K. R. and Harrison, B. D., *CMI/AAB Descriptions of Plant Viruses*, 1985, **297**, 1.
4. Ikegmi, S., Haber, S. and Goodman, R. M., *Proc. Natl. Acad. Sci. USA*, 1981, **78**, 4102.
5. Moringa, T., Ikegami, M., Arai, T., Yazaki, K. and Miura, K., *J. Gen Virol.*, 1988, **69**, 897.
6. Draper, J. and Scott, R., in *Plant Genetic Transformation and Gene Expression, A Laboratory Manual* (eds. Draper, J., Scot, R., Armitage, P. and Walden, R.), Blackwell Scientific Publications, Oxford, 1988, p. 201.
7. Hamilton, W. D. O., Bisaro, D. M. and Buck, K. W., *Nucl. Acids Res.*, 1982, **10**, 4901.

Received 7 September 1991; accepted 24 October 1991

Cloning and use of low copy sequence genomic DNA for RFLP analysis of somaclones in mustard (*Brassica juncea* (L.) Czern and Coss)

T. Mohapatra, R. P. Sharma and V. L. Chopra
Biotechnology Centre, Indian Agricultural Research Institute, New Delhi 110 012, India

***Brassica juncea* (L.) Czern and Coss genomic DNA fragments of 0.5 to 2.5 kb size, obtained by Pst I digestion were cloned into the pUC18 plasmid vector. Screening of the partial library by dot blot hybridization using labelled total genomic DNA as probe revealed that about 45% of the clones carry low copy number DNA sequences. Using some of these clones as probes, DNA polymorphism could be detected among somaclones of *B. juncea* cv. Varuna.**

RESTRICTION fragment length polymorphism (RFLP) analysis is used for tagging genes controlling complex quantitative traits, varietal identification and establishing phylogenetic relationship. Successful application of the technique, however, requires a large number of low copy DNA sequences, used as probes in Southern hybridization. To meet this need, partial libraries of random low copy genomic DNA sequences have been constructed in several plant species[1–4]. The methylation-sensitive Pst I restriction enzyme has played a key role in this context. Pst I can specifically access the low copy sequences which are generally un- or undermethylated. Figdore *et al.*[5] reported that more than 75% of Pst I-generated clones carry low copy sequences in the diploid *Brassica* species — *B. oleracea* and *B. campestris*. In this communication, we report construction of a partial Pst I genomic library of low copy sequence genomic DNA and its application in RFLP analysis of somaclones in the alloploid *Brassica juncea* (L.) Czern and Coss.

Genomic DNA isolated from 4-day-old etiolated seedlings of *B. juncea* cv. Varuna, following Dellaporta *et al.*[6], was purified by CsCl density gradient centrifugation, digested with Pst I and electrophoresed on a 1% agarose gel. DNA fragments, 0.5 to 2.5 kb in size, were electroeluted from the gel and purified by phenol–chloroform extraction[7]. Though the DNA was completely digestable with methylation-insensitive enzymes such as Hind III and Msp I, it was observed that even after overnight digestion with Pst I, a major portion of genomic DNA remained uncut, suggesting that *B. juncea* genome is highly C-methylated. This resulted in very low yield (3.6 μg out of 40 μg of DNA digested) of desired fragments. Besides, five fluorescing bands seen (Figure 1) within the desired size range indicated that some repetitive DNA sequences are still present in the eluted fraction.

Figure 1. Gel photograph showing Pst I restriction of *B. juncea* genomic DNA: Lanes 2 and 3 carry overnight Pst I digest. Control unrestricted DNA and λ Hind III digest as molecular weight marker are in lanes 1 and 4 respectively.

DNA fragments, thus obtained, were cloned in plasmid pUC18. For this the plasmid DNA was linearized by Pst I and treated with alkaline phosphatase to prevent self-ligation. The vector and the eluted DNA fragments were mixed in molar ratios of 1:4, 1:6 and 1:8 for overnight ligation at 15°C. Competent cells of *E. coli* JM107 transformed[8] by each of these ligated DNA samples, yielded a total of 92, 148 and 261 recombinant clones respectively. Pst I restriction analysis of 25 randomly chosen clones confirmed the presence of cloned DNA.

For identifying low copy clones, plasmid DNA isolated from all the 501 clones was dot blotted[9] onto nitrocellulose and probed with nick translated α-^{32}P labelled total genomic DNA. In the autoradiograms (Figure 2) the extent of hybridization in different clones, as revealed by visual observations and laser beam densitometric scanning, was found to vary. This variation could be due to the fact that eukaryotic genomes contain about 50% or more of repetitive DNA. Therefore, the amount of α-^{32}P dNTP incorporated in the repetitive sequences during nick translation of total genomic DNA and the extent of its hybridization with the dot blotted recombinant plasmids are expected to be higher than the single/low copy sequences. Consequently, the clones showing very high hybridization signal intensity are likely to be highly repetitive, while those having intermediate and low or as much signal intensity as the control pUC18 are likely to be moderately repetitive and single/low copy respectively. Based on this rationale, 34 (6.8%) clones were identified as highly repetitive, 239 (47.7%) as moderately repetitive and 228 (45.5%) as low copy. This proportion of low copy clones is, however, lower than that reported by Figdore *et al.*[5] in the diploid *Brassica oleracea* ssp. *capitata* (76.4% low copy of a total of 161 clones) and in *B. campestris* ssp. *pekinensis* (75% of a total of 140 clones).

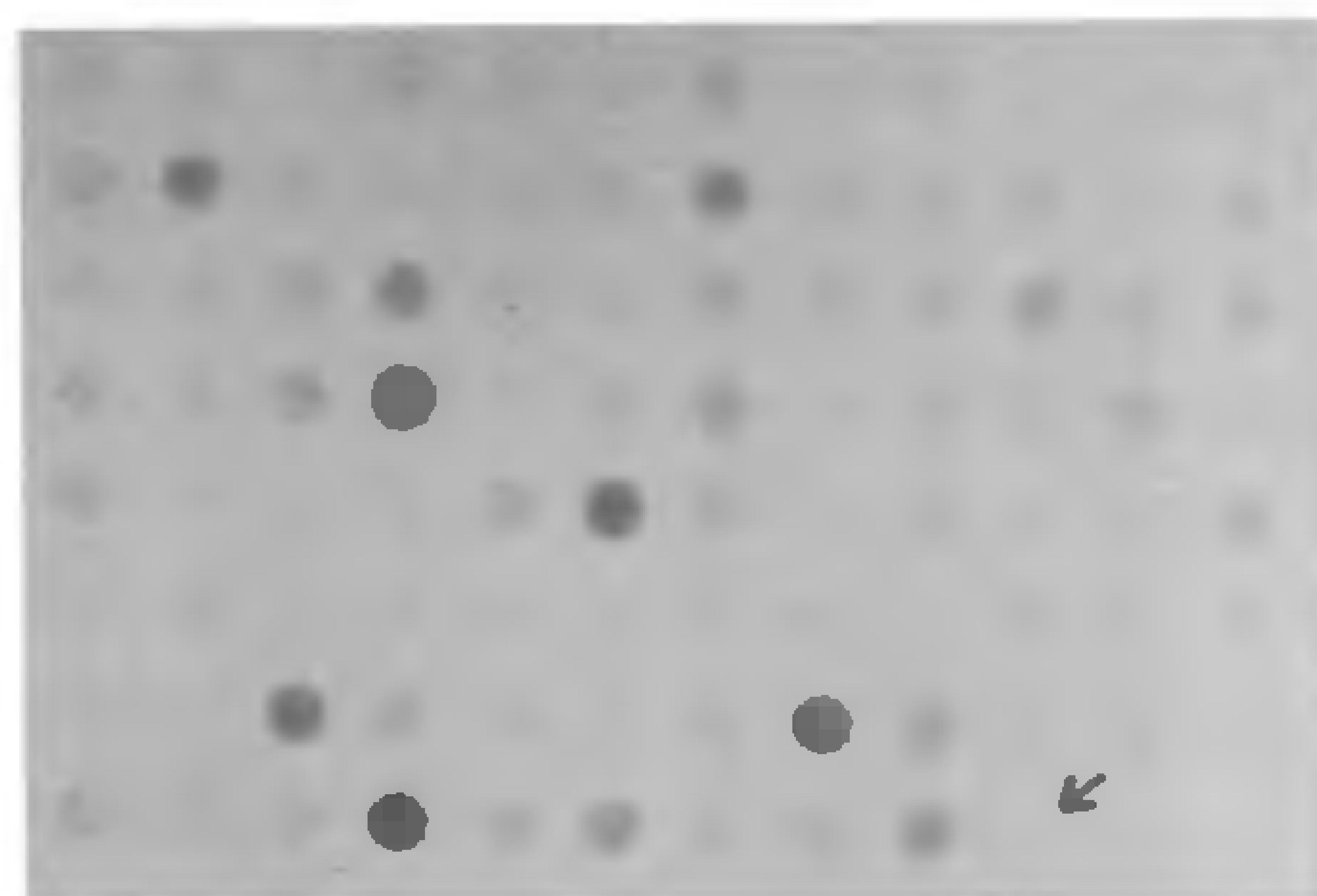

Figure 2. Autoradiogram showing differential hybridization signal of different clones with radiolabelled total genomic DNA: Arrow indicates the extent of non-specific hybridization with the control PUC 18 DNA.

The amphidiploid nature of *Brassica juncea* might explain low proportion of low copy sequence clones in our study. Recent reports on hybridization of RFLP markers with *B. oleracea* monosomic alien chromosome addition lines have indicated occurrence of extensive intragenomic DNA duplications[10]. Besides, many of the low copy sequence clones from the Pst I library of *B. oleracea* ssp *capitata* have been shown to hybridize with multiple DNA fragments at intraspecific[11] as well as interspecific levels[12]. It is possible that a low copy DNA sequence from a diploid which is accessible to Pst I, will behave as multicopy or be moderately repetitive in an amphidiploid, depending on the extent of intra and inter-genomic DNA duplications. Presence of such sequences in the amphidiploid would obviously result in low proportion of low copy sequence clones in the Pst I library.

A set of low copy clones thus obtained was employed for assessing DNA polymorphism in the parental variety Varuna and its six somaclones identified phenotypically in replicated trials as early maturing-1, early maturing-2, late maturing, tall-1, tall-2 and productive, in their selfed R_6 generation. For this, genomic DNA was isolated following Saghai-maroof *et al.*[13] with the modification that instead of 15 volumes of 1 × CTAB extraction buffer, 5 volumes of 2 × buffer was used for fresh leaf material, ground in liquid nitrogen. This

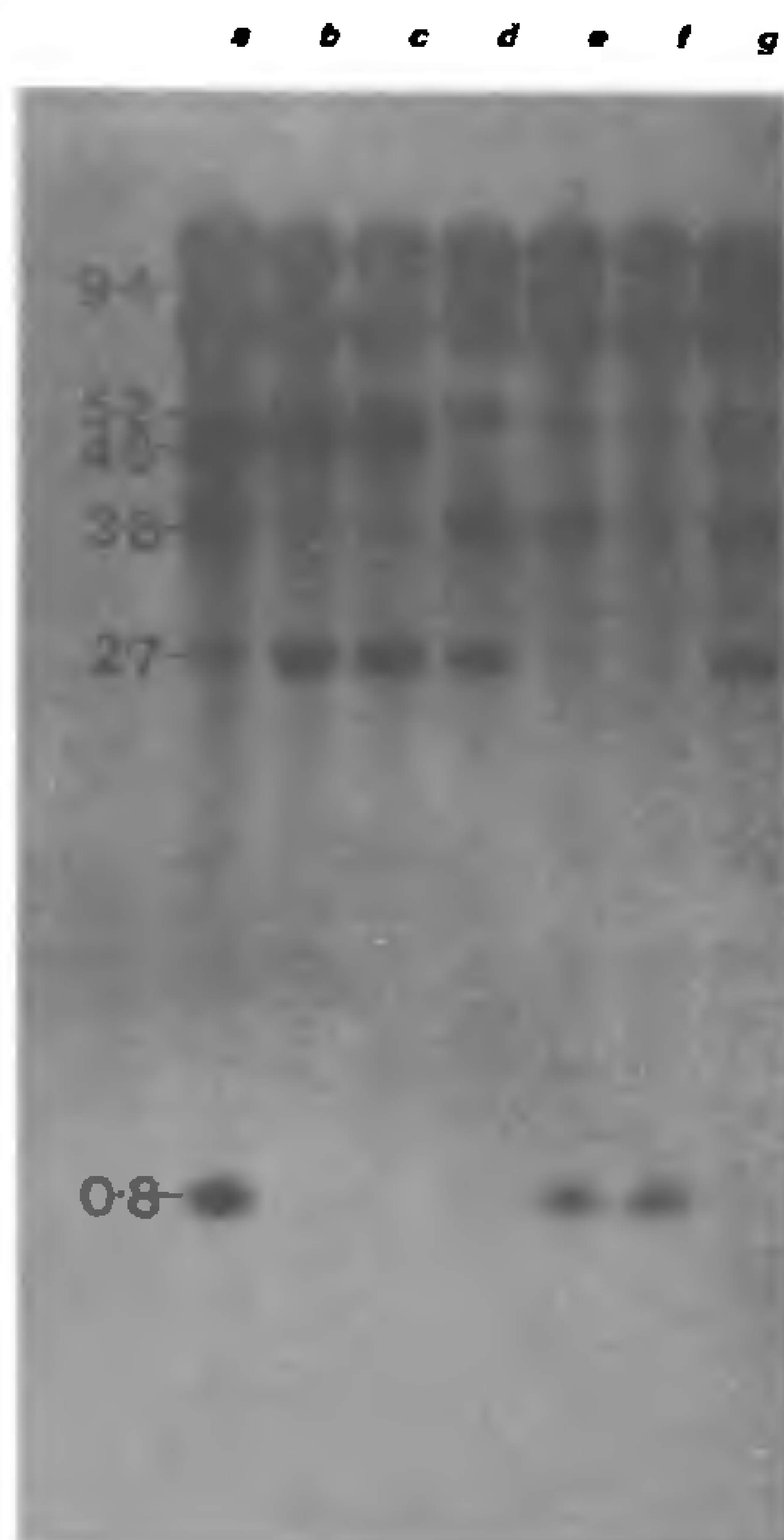

Figure 3. Autoradiogram showing DNA polymorphism among somaclones of *B. juncea*: *a*, Parent variety Varuna; *b*, Early maturing-1; *c*, Early maturing-2; *d*, Late maturing; *e*, Tall-1; *f*, Tall-2; *g*, Productive. Size of the hybridizing DNA fragments in kb are indicated on left hand margin. The clone BJG 431 was used as the probe.

protocol was used to eliminate polysaccharides generally known to affect the quality and thus digestability of DNA. On further purification by CsC1 density gradient centrifugation, DNA was digested with Hind III, Eco RI and Eco RV (Promega), electrophoresed and transferred onto Gene-Screen plus nylon membrane (DuPont) using an LKB vacuum blotting unit. Hybridization was carried out following Kochert *et al.*[14] using α-^{32}P labelled recombinant plasmids as probes. Out of 15 probe–enzyme combinations tried 10 combinations detected polymorphism. Clone BJG 431 detected DNA polymorphism among all the selected somaclones (Figure 3). The two early maturing somaclones could not be distinguished from each other in spite of their phenotypic differences but all the six could be differentiated from the parent variety. The variation in Southern hybridization pattern indicated occurrence of DNA rearrangements and/or point mutations in the selected somaclones. These somaclones are in the advanced generation of selfing and have attained uniformity for the selected traits. The observed DNA alterations, therefore, represent stable and heritable somaclonal variation at molecular level.

1. McCouch, S. R. *et al.*, *Theor. Appl. Genet.*, 1988, **76**, 815.
2. Helentjaris, T., Slocum, M., Wright, S., Schaefer, A. and Nienhuis, J., *Theor. Appl. Genet.*, 1986, **72**, 761.
3. Landry, B. S., Kesseli, R. V., Farrora, B. and Michelmore, R. W., *Genetics*, 1987, **116**, 331.
4. Gebhardt, C. *et al.*, *Theor. Appl. Genet.*, 1989, **78**, 65.
5. Figdore, S. S., Kennard, W. C., Song, K. M., Slocum, M. K. and Osborn, T. C., *Theor. Appl. Genet.*, 1988, **75**, 833.
6. Dellaporta, S. L., Wood, J. and Hicks, J. B., *Plant Mol Biol. Rep.*, 1983, **1**, 19.
7. Maniatis, T., Fritsch, E. F. and Sambrook, J., *Molecular Cloning*, Cold Spring Harbor Laboratory, 1982.
8. Davis, L. G., Dibner, M. D. and Battey, J. F., *Basic Methods in Molecular Biology*, Elsevier, New York, 1986.
9. Mason, P. J. and Williamus, J. G., in *Nucleic Acid Hybridization* (eds. Hames, B. D. and Higgins, S. J.), IRL Press, Oxford, 1988, p. 113.
10. McGrath, J. M., Quiros, C. F., Harada, J. J. and Landry, B. S., *Mol. Gen. Genet*, 1990, **223**, 198
11. Slocum, M. K., Figdore, S. S., Kennard, W. C., Suzuki, J. Y. and Osborn, T. C., *Theor. Appl. Genet.*, 1990, **80**, 57.
12. Song, K. M., Osborn, T. C and Williams, P. H., *Theor. Appl. Genet.*, 1988, **75**, 784.
13. Saghai-maroof, M. A., Soliman, K. M., Jorgensen, R. A. and Allard R. W., *Proc. Natl. Acad. Sci. USA*, 1984, **81**, 8014.
14. Kochert, G., Tanksley, S. D. and Prince, J. P., *Training Course Laboratory Manual*, Rockefeller Foundation, 1989.

Received 4 September 1991; revised accepted 24 October 1991

In vitro plant regeneration from phylloid flowers of niger (*Guizotia abyssinica* cass)

M. Sujatha

Genetics and Plant Breeding Section, Directorate of Oilseeds Research, Rajendranagar, Hyderabad 500 030, India.

The occurrence of phyllody disease in niger is reported. An *in vitro* regeneration technique for production of whole plantlets from phyllody parts in niger is described. Antibiotic sensitivity test with tetracycline caused remission of symptoms indicating perpetuation of the causative organism namely the mycoplasma-like organisms (MLO). The technique could facilitate rapid screening of germplasm and breeding materials against MLO's and pave the way for identification of sources of resistance.

PLANT tissue culture has potential application in the development of disease-resistant crops. Besides, the system also enables rapid screening of a large number of genotypes year-round within a relatively small space. The crops affected by phyllody disease which is caused