SOMACLONAL VARIATION IN BLACK PEPPER (*Piper nigrum* L.)

BY

M. R. SHYLAJA

THESIS

Submitted in partial fulfilment of the
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Department of Plantation Crops and Spices
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DECLARATION

I hereby declare that this thesis entitled **"Somaclonal variation in black pepper (*Piper nigrum* L.)"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title of any other University or Society.

Vellanikkara

8/3/96

SHYLAJA, M.R.

CERTIFICATE

Certified that the thesis entitled **"Somaclonal variation in black pepper (*Piper nigrum* L.)"** is a record of research work done independently by **Smt.M.R. Shylaja** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

G. Sreekandan 8/3/96

Dr.G. SREEKANDAN NAIR
Chairman of Advisory Committee
Professor and Head
Department of Plantation Crops and Spices
College of Agriculture
Vellayani

Vellanikkara
8-3-'96

CERTIFICATE

We the undersigned, members of the Advisory Committee of **Smt.M.R. Shylaja**, a candidate for the degree of Doctor of Philosophy in Horticulture, agree that the thesis entitled **"Somaclonal variation in black pepper (*Piper nigrum* L.)"** may be submitted by Smt.M.R.Shylaja in partial fulfilment of the requirements for the degree.

Dr.G.SREEKANDAN NAIR
Professor and Head
Department of Plantation Crops and Spices
College of Agriculture, Vellayani
(Chairman)

Dr.P.A. NAZEEM
Associate Professor
College of Horticulture
Vellanikkara
(Member)

Dr.V.K. MALLIKA
Associate Professor
College of Horticulture
Vellanikkara
(Member)

Dr.P.A. WAHID
Associate Dean
College of Agriculture
Nileshwar, Kasargode
(Member)

Dr.JAMES MATHEW
Professor and Head
Department of Plant Pathology
College of Horticulture
(Member)

EXTERNAL EXAMINER

Dr. C. PADMANABHAN
CPMB, TNAU.

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SHYLAJA, M.R.

To my beloved parents
and
to the loving memories
of my father-in-law
and mother-in-law

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ABBREVIATIONS

BAP	- benzyl amino purine
IAA	- indole-3-acetic acid
IBA	- indole-3-butyric acid
2,4-D	- 2,4-dichloro phenoxy acetic acid
h	- hour
mg l^{-1}	- milligram per litre
MS	- Murashige and Skoog's (1962) medium
v/v	- volume in volume
Gy	- Grays
kR	- kilo Rad
cv.	- cultivar
ie.	- that is
@	- at the rate of
μM	- micro molar

Introduction

INTRODUCTION

Black pepper (*Piper nigrum* L.), the king of spices popularly known as the black gold, is the most important export oriented commodity and foreign exchange earner among the Indian spices. In India, pepper is grown in an area of 1,83,805 ha with a production of 51,604 t (Spices Board, 1994). Of the total 607 crores of export earnings from an export of 1.54 lakh t of spices during 1994-'95, black pepper accounted for 23 per cent in quantity and 37 per cent in value.

The Indian pepper enjoyed monopoly in the world market till the turn of the 19th century when other countries like Indonesia and Brazil entered the pepper trade. The productivity in India is the lowest compared to other pepper producing countries such as Indonesia, Malaysia and Brazil.

Ravages due to diseases, particularly the most devastating *Phytophthora* foot rot disease caused by *Phytophthora capsici* is one of the major constraints in production. Not only the production but also the wealth of genetic diversity available for the crop is plagued with this dreadful disease. Many of the valuable genotypes are lost every year from the gene pool due to this serious malady.

The crop loss due to *Phytophthora* foot rot disease has been estimated by different workers. Samraj and Jose (1966) and Nambiar and Sarma (1977) reported 20-30 per cent of vine death in Kannur and Kozhikkode districts. An annual loss of 905 and 119 t of black pepper respectively was reported from these districts by Balakrishnan *et al.* (1986) and Anandaraj *et al.* (1988).

A recent survey in Kannur district showed that the death of vines due to *Phytophthora* foot rot followed a parabolic trend with maximum incidence during September. The cultivars like Balankotta, Kalluvally, Uthirankotta, Arakkulam-munda and Arikotta appeared to be more tolerant to the disease when compared to Karimunda and Panniyur-1. The death due to disease in tolerant cultivars was just half of that observed in Karimunda and Panniyur-1 (Kerala Agricultural University, 1993).

Detailed symptomatology of the disease has been described by Mammootty (1978) and Sarma and Nambiar (1982). The fungus infects all parts of black pepper but the collar and root infections are most fatal and the infected vine succumbs in 10-20 days. Integrated disease management involving chemical, cultural and biological methods besides host resistance is reported to be the ideal strategy to combat the disease (Sarma *et al.*, 1988).

The conventional disease resistance breeding mainly focussed on screening the available germplasm, open pollinated and irradiated seedling progenies for their relative degree of resistance/tolerance to the disease. Screening the available genetic diversity of black pepper for resistance to *Phytophthora* foot rot was done by several workers (Holliday and Mowat, 1963; Leather, 1967; Kueh and Khew, 1980b; Vilasini, 1982, Sarma *et al.*, 1982) and none of the cultivated types were found to be resistant. Sarma *et al.* (1982) found no resistance in wild types also, when 41 cultivars and 73 wild types of *Piper* spp. were screened for resistance to *Phytophthora* foot rot. However, cultivars like Narayakodi, Kalluvally, Uthirankotta and Balankotta were found to be tolerant in the study. Sarma *et al.* (1991) further reported that since high degree of resistance is lacking in the available germplasm,

available tolerance should be utilised in the hybridisation programmes and the immunity of *Piper colubrinum* to *Phytophthora capsici* should be exploited.

Exploitation of the *in vitro* culture induced genetic variation (somaclonal variation) as such and induction of variation through *in vitro* techniques will be thus important in disease resistance breeding in a crop like black pepper, where there is no resistance in the cultivated types and the available resistance in wild species is difficult to transfer. The desirable plant types thus produced could either be directly exploited or later incorporated into other high yielding types.

Somaclonal variation has been observed in many monocots and dicots, sexually as well as asexually reproducing species (Larkin and Scowcroft, 1981). Somaclonal variants were found to occur at high frequencies, the frequency of variation has been estimated to be as high as 30-40 per cent for the number of plants showing some type of variation and from 0.2 to almost 3 per cent for variation in a particular trait (Evans *et al.*, 1984).

The causes of somaclonal variation are thought to be due to a combination of factors. Some of the variability is due to pre-existing mutations in cells of the explant material (Lorz, 1984 and Orton, 1984). A large part of the variation is induced during the culture cycle and this variation is attributed to chromosomal abnormalities commonly observed in cultured cells. Ploidy changes and changes including translocations, deletions, amplifications and point mutations occur in culture cycle (Larkin *et al.*, 1984). Also, changes occur both in single gene and in polygenic traits (Evans and Sharp, 1983; Evans *et al.*, 1984; Larkin *et al.*, 1984) and in both organelle and nuclear genomes (Mc Nay *et al.*, 1984).

Many factors are found to influence the rate of somaclonal variation. They include growth regulators (Evans, 1988; Griesbach *et al.*, 1988; Shoemaker *et al.*, 1991), cultivar (Kurtz and Lineberger, 1983, Hwang and Ko, 1986), cultivar age (Shepard *et al.*, 1980), ploidy level (Heinz and Mee, 1969; Bingham and Mc Coy, 1986), explant source (Shepard *et al.*, 1980; Gui *et al.*, 1993; Tsai *et al.*, 1992), length of time *in vitro* (Skirvin *et al.*, 1994), proliferation rate (Smith and Drew, 1990) and cultural conditions (Skirvin *et al.*, 1994).

Somaclonal variation has been extensively exploited for isolating disease tolerant/resistant types in several crop species. The isolation of disease resistant/ tolerant lines was attempted with and without applying *in vitro* selection pressure with toxic metabolites of the pathogen. The possibility of utilising somaclonal variation as a valuable adjunct to disease resistance breeding was investigated by several workers like Carlson (1973) in tobacco, Heinz *et al.* (1977); Liu (1981) and Larkin and Scowcroft (1983) in sugarcane, Miller *et al.* (1985) in tomato, Thomson *et al.* (1986) in potato and Heath-Pagliuso *et al.* (1989) in celery.

Similar reports on isolation of disease tolerant/resistant types through *in vitro* techniques are scanty in black pepper. However, callus initiation and subsequent regeneration is possible. Good callus growth from various explants of *in vitro* seedlings and mature vines of black pepper was reported by Mathews and Rao (1984) and Rajmohan (1985) respectively. Successful callus mediated organogenesis from *in vitro* seedling explants of black pepper was reported by Nazeem *et al.* (1990). Indirect organogenesis from explants of mature vines of black pepper was further reported by Nazeem *et al.* (1993) and Philip *et al.* (1995).

The present investigations were thus aimed at exploiting somaclonal variation in black pepper and developing screening procedures for *Phytophthora* foot rot resistance/tolerance using tissue culture techniques.

Review of Literature

REVIEW OF LITERATURE

Phytophthora foot rot disease of black pepper caused by *Phytophthora capsici* is the most dreadful disease where ever the crop is cultivated. Many of the valuable genotypes are wiped out every year due to this serious malady. The conventional breeding programmes so far carried out for the identification of resistant genotypes and development of improved types incorporating the factors of resistance were found unsuccessful.

The available cultivars of black pepper and related species of *Piper* were screened for resistance to *Phytophthora* foot rot by several workers (Holliday and Mowat, 1963; Ruppel and Almeyda, 1965; Leather, 1967; Turner, 1973; Sarma *et al.*, 1982; Kueh and Khew, 1980b; Vilasini, 1982) and found that none of the cultivated types were resistant to this disease even though some wild species like *P. colubrinum* and *P. obliqum* showed resistance.

Therefore *in vitro* culture induced genetic variation constitute an important source of variability for the improvement of black pepper and hence the search for somaclonal variants and isolation of tolerant/resistant plant types is of great significance.

The possibility of using cell and tissue culture techniques in disease resistance breeding was investigated by several workers and excellent reviews on the subject have been made by Brettel and Ingram (1979); Daub (1986) and van den Bulk (1991).

Helgeson and Haberlach (1980) and Ingram (1980) mentioned the advantages and disadvantages of *in vitro* cell and tissue culture technique in screening for resistance to pathogens. The most important advantages are freedom from the effects of climate and other natural environment which makes it easier to measure slight quantitative differences in inherited resistance, the ability to handle large numbers of individuals in a very small space, the ability to work with microspores and haploids, the simpler genomes which allow the uncovering of recessive traits and additive characters with in a small population and elucidation of the basic mechanism of host pathogen interaction in a wide range of plant diseases caused by fungi, bacteria and viruses.

There are however some disadvantages of tissue culture systems for resistance studies, protective tissues such as cuticle may be absent; the tissues are often actively growing and therefore resemble only meristematic area of the plant; sometimes resistance is not expressed in culture or biochemical events associated with resistance may differ between plants and cells/calluses.

To use this technique successfully there should be a basic knowledge of the sources of variability in cell and tissue culture, different culture systems, methods of screening variants, the technique of regenerating plants and accurately screening the regenerants.

For *in vitro* selection system to be effective a screening agent should be incorporated in the culture to discriminate the susceptible cells/calluses. The selective agent should be a determinant of the disease and should act at the level where selection will be carried out.

There are no reports available on the use of *in vitro* techniques in disease resistance breeding in black pepper. Hence, the present review deals with the application of *in vitro* techniques for disease resistance breeding in other crops. The review focuses the *in vitro* screening methods using pathogens and pathogen metabolites, screening of somaclonal variants *in vivo* at whole plant level, effect of gamma irradiation on callus growth, organogenesis and recovery of disease resistant mutants, production of toxic metabolites by *Phytophthora* spp. and partial purification of toxic metabolites of *Phytophthora* spp.

2.1 *In vitro* screening for resistance to diseases

2.1.1 Pathogen as the screening agent

The use of pathogen itself as the screening agent is considered as the most direct approach. Since uniformity of infection could not be achieved, this method is rarely used. However, some attempts have been made to use pathogen as the selecting agent to study the host-pathogen interaction at cellular level and for *in vitro* screening for resistance to diseases.

2.1.1.1 Screening for viral diseases

Shepard (1975) isolated protoplasts from tobacco plants systemically infected with potato virus x and indexed 4000 plants regenerated from these protoplasts. Approximately 8 per cent of the protoplast derived plants were virus free and one of these plants showed resistance to virus.

Murakishi and Carlson (1982) isolated protoplasts from systemically infected haploid *Nicotiana sylvestris* plants. They used a yellow strain of tobacco

mosaic virus (TMV-Flavum) and gave high temperature, high humidity and dim light that allowed uniform infection of leaf cells. Leaf strips from infected plants grown aseptically were plated on a medium containing high concentrations of cytokinin and grown under high light intensity. The rapidly growing green calli could be separated from the slow growing yellow virus infected calli. Out of the 763 green calli, seven regenerants were virus resistant. The resistance was transmitted to progeny and was expressed as a reduction in virus multiplication and movement resulting in a three to eight weeks delay in symptom development.

Infecting the protoplasts and screening the regenerated plants for virus resistance was also reported by Harrison and Mayo (1983) and Murakishi *et al.* (1984) in tobacco.

2.1.1.2 Screening for fungal diseases

Varying results have been obtained when pathogens other than viruses are used to select resistant cells.

Helgeson *et al.* (1972) induced callus cultures from resistant and susceptible plants to race 0 of *Phytophthora parasitica* var. *nicotianae*. The pathogen colonized less rapidly in calluses of resistant plants while colonized extensively in the calluses of susceptible plants.

In a more detailed study Helgeson *et al.* (1976) compared the reaction of rooted cuttings and pith callus tissues from 185 tobacco plants to *Phytophthora parasitica* var. *nicotianae*. In each case, plants that produced resistant cuttings produced only resistant calluses and plants that produced susceptible cuttings produced only susceptible calluses. The single dominant genetic factor which

conferred disease resistance to intact tobacco plants was expressed in tobacco pith callus cultures.

Ingram (1976) studied the expression of resistance to *Phytophthora infestans* in potato tissue cultures. The potato varieties Orion, Vester, Torch and Pentland Beauty and related species like *Solanum demissium* and *S. stoloniferum* did not support growth of *P. infestans* race 4 while the susceptible potato variety Majestic supported good growth. The potato variety Orion is susceptible to race 1 but resistant to race 4. Hence considerable mycelial development occurred in the case of race 1 and only rudimentary growth was observed in the case of race 4. The variety Majestic was susceptible to both the races and hence good growth of both the races was observed in the variety.

The effects of varying cytokinin and auxin concentrations on resistance of tobacco tissue cultures to race 0 of *Phytophthora parasitica* var. *nicotianae* were studied by Haberlach *et al.* (1978). With 1 μM kinetin and either 11.5 μM IAA or 1 μM 2,4-D tissue from resistant cultivars exhibited a 'hypersensitive' reaction to zoospores of the fungus and subsequently colonized slightly. With higher levels of cytokinin (10 μM kinetin) in susceptible and resistant cultivars the hypersensitive reaction did not occur and tissues were heavily colonized. BAP and kinetin were particularly effective in eliminating both the hypersensitive reaction and disease resistance. Zeatin and 6 (3 methyl 2-butenylamino) purine were less effective. Increases in indole acetic acid levels reversed the effects of high cytokinin concentrations. Thus the balance of phytohormones apparently controls the host response to the fungus. Thus the resistance or susceptability can be studied without changing the host or fungal pathogen.

Holliday and Klarman (1979) used soyabean callus culture for understanding the resistance mechanism of *Phytophthora megasperma* at cellular level. Calluses derived from soyabean plants of resistant cultivar 'Cutler 71' and susceptible cultivar 'Cutler' were screened by inoculating zoospores of *Phythophthora megasperma* var. Sojae (Pms). Cutler 71 calluses were colonized less than those of cutler when both were grown on medium containing 6 or 10 mg of 2,4-D/l and incubated at 16 or 20°C prior to and following inoculation.

Sacristan and Hoffmann (1979) infected callus cultures of halploid *Brassica napus* with resting spores of *Plasmodiophora brassicae* to select cell colonies resistant to the pathogen. The pathogen was able to penetrate into cells of unorganized callus but it completed its life cycle in cultures with a certain degree of differentiation. The appearance of green embryos from necrotic tissue was first interpreted as a possible sign of resistance. However, later studies by Sacristan (1985) demonstrated that the infection and its phenotypic effects were unspecific since they also occur in cultures of rape lines with specific resistance against the pathotype.

Transmission and scanning electron microscopy was used by de Zoeten *et al.* (1982) to determine the response of tobacco callus tissue to inoculation with *Phytophthora parasitica* var. *nicotianae*. Calluses resistant to race 0 and susceptible to race 1 and susceptible to race 0 and resistant to race 1 was used. Within three h after inoculation zoospores from compatible and incompatible races had encysted and germinated, the germ tubes of both races penetrated callus cells. By 24 h, when a hypersensitive reaction (HR) was clearly evident in the incompatable combination, almost all the cells in the HR area had collapsed, where as most cells in infected

areas on callus showing the compatible reaction were still turgid. After 48 h, hyphae of the compatible fungus had penetrated < 50 cell layers, where as those of the incompatible fungus were limited to five to eight cell layers.

Sacristan (1982) could select resistant mutants by inoculating rape callus tissues with *Phoma lingam* spores. The embryogenic calluses which were free of infection by the fungus were considered resistant. However, the resistance of the regenerated plants was less.

Prameela Devi (1983) and Prameela Devi and Narayanaswamy (1988) reported a method of culturing *Erysiphe polygoni* on callus tissue of blackgram and green gram. Significant reduction in total carbohydrate content and an increase in total soluble sugars, reducing sugars and non reducing sugars were observed in the calli of susceptible varieties following inoculation with pathogen. The calli from resistant varieties showed only marginal variation in total carbohydrate content and the sugars marginally increased and then decreased following inoculation.

Vidyasekharan *et al.* (1984) observed that *Helminthosporium oryzae* grew slowly in calluses of incompatible rice varieties but at a faster rate on calluses of compatible varieties.

Miller *et al.* (1984) studied resistance to *Phytophthora megasperma* f. sp. *medicaginis* (Pmm) and *P. megasperma* f. sp. *glycinea* (Pmg) in alfalfa callus cultures. Hyphae of Pmm completely covered four to six week old calli from a plant susceptible to Pmm (M 269) with in five days after incubation but growth was limited on calli derived from a plant resistant to Pmm (M 194). They further observed that both callus morphology and expression of disease resistance

were influenced by the concentration of kinetin in the growth medium, as the kinetin concentration decreased the consistency of the calli appeared more fluid and they were more susceptible to both Pmm and Pmg. A kinetin concentration of atleast 2.0 mg/l of growth medium was necessary for clear differentiation of resistant and susceptible genotypes.

Prasad *et al.* (1984) isolated plants of pearl millet resistant to downy mildew (*Sclerospora graminicola*) by regenerating plants from immature inflorescence explants pre infected with the fungus.

Utkhede (1986) reported *in vitro* screening of the world apple germplasm collection for resistance to *Phytophthora cactorum* Crown rot. *In vitro* dormant excised twigs established from 516 accessions of the USDA world germplasm collection were inoculated with *P. cactorum*. The lesion length was used as a measure of relative resistance. Eighty two plant introduction cultivars were significantly resistant compared to one hundred and sixty two very susceptible cultivars, including 'M_9' moderately resistant root stock. Of the 82 cultivars, 16 were considered to be very resistant while 66 were moderately resistant. These results indicate variation in resistance and suggest the possibility of breeding resistant root-stocks to control the disease.

Callus cultures initiated from Australian native and horticultural species of plants like *Acacia, Eucalyptus, Lupinus* and *Lycopersicon* with varying degrees of field resistance or tolerance to the fungal pathogen *Phytophthora cinnamomi* were screened for the expression of resistance to the pathogen by McComb *et al.* (1987). The extent of hyphal growth on callus was correlated with the susceptability of the plant from which the callus was derived. The amount of callose formed in the

cultured cells in response to the pathogen was correlated with the degree of field resistance. Callose, detected by fluorescence with decolourised aniline blue was present as large deposits in callus derived from the resistant species while no callose was observed in infected callus from susceptible plants.

Ruma Palit and Reddy (1987) inoculated spore suspension of *Pyricularia oryzae* on irradiated calli of a susceptible variety and incubated for two weeks. Portions of calli were seen growing and free from mycelial growth. Out of 104 plantlets regenerated from such calli eight exhibited resistance.

A procedure was developed by Jang and Tainter (1990) which demonstrated the expression of differential resistance in pine callus tissues to *Phytophthora cinnamomi*. Callus tissues were maintained on a modified MS medium with 10^{-5} M 2,4-D and inoculated with hyphae of *P. cinnamomi* at 26°C in the dark. The number of intracellular hyphae was used as an index of resistance. Loblolly and Loblolly x short leaf pine hybrids were found to be more resistant than short leaf and virginia pine.

Jang and Tainter (1991) further studied the growth regulator effects on callus and fungus separately and then together in order to refine the resistance screening protocol. They found that callus tissues maintained on a modified MS medium with 10^{-5} M 2,4-D at 26°C in the dark optimized the expression of differential resistance when inoculated with hyphae of *P. cinnamomi* and high concentration of 2,4-D (5×10^{-5} M) inhibited the growth of *P. cinnamomi*.

The micropropagated shoots of *Carica papaya* were inoculated *in vitro* through a stem wound with sporangial suspension (1.2×10^{4} sporangia ml^{-1}) of

Phytophthora palmivora by Sharma and Skidmore (1988). The symptoms exhibited by the shoots *in vitro* were similar to the infection of the whole plant in the field. The time taken for the host tissue to become brown and to wilt and the time of sporulation of the pathogen were recorded for each shoot of four varieties of papaya challenged with ten isolates of *P. palmivora*. Significant differences were observed between host-pathogen combinations for the variables and host specificity was detected amongst the isolates of *P. palmivora*. They concluded that similar selection *in vitro* for disease resistance will be useful in papaya breeding.

Callus derived single cells or small clusters in celery were cocultured with a hypovirulent and normal isolate of *Septoria apiicola* by Evenor *et al.* (1994). Some of the plants regenerated from surviving cells showed tolerance to *S. apiicola* in green house tests.

2.1.1.3 Screening for Bacterial diseases

Rehbein (1983) reported that out of 3,016 potato calli treated with *Erwinia carotovora* 312 survived. Out of these 83 calli formed plantlets, 23 of which were more resistant to *E. carotovora* than control and one clone expressed resistance at the tuber level also.

Sun *et al.* (1986) inoculated 365 calluses from dehulled grains for susceptible rice variety with the bacterial blight pathogen, *Xanthomonas compestris* pv. *oryzae*. Out of the 63 calluses showed sectional proliferation 45 plants could be regenerated. All but one were resistant to the disease. The progenies of the resistant plants carried the resistance for three more generations tested.

Dunbar and Stephen (1988) developed a rapid *in vitro* assay for screening geranium somaclones for resistance to bacterial blight. Shoots from the callus cultures were placed in tubes containing 15 ml of Hogland's solution solidified with 0.7 per cent agar and inoculated *in vitro* by rubbing the upper surface of individual leaves with cotton swab dipped in 10^7 CFU/ml suspension of *Xanthomonas campestris* pv. *pelargonii* cells. Three weeks after, seed geranium somaclones regenerated from susceptible cultivars showed significantly lower survival rate than 'regal' geranium somaclones used as resistant controls.

Achieving uniform exposure is not the only concern when dealing with pathogen selected cells in culture, differential response at the cellular level and whole plant level is another major consideration (Daub, 1986). Uchiyama and Ogasawara (1977) and Uchiyama (1983) reported the increase in susceptibility of rice callus to several pathogens was due to the lack of cuticular wax on calluses and lack of lignification and free phenol accumulation on calluses.

2.1.2 Toxic metabolites of Pathogen as screening agent

The efficiency of a selection procedure may be increased by the application of selection pressure *in vitro* to cells/protoplasts or calluses. The most common method for selecting for disease resistance in culture has been the use of pathogen toxic metabolites as the selecting agent. This method overcomes one of the problems encountered with pathogen selection since the cultured cells can be exposed uniformly to the screening agent (Daub, 1986). The selective agent should be a determinant of the disease and it should act at the level where selection is carried out.

By exposing the cultured cells to partially purified toxins or culture filtrates containing the toxic metabolites disease resistant plants have been regenerated in many crops.

2.1.2.1 Culture filtrates of the pathogen as the screening agent

Potato

Behnke (1979) selected dihaploid calli of *Solanum tuberosum*, resistant to the culture filtrates of four patho types of *Phytophthora infestans*. The resistance was found to express in the regenerants as well as in the calli induced from leaf segments of the resistant regenerants, when tested with the culture filtrate of the pathogen.

Behnke (1980) induced lesions on leaves of the regenerants using sporangial suspensions (250 sporangia/25 μl) of *Phytophthora infestans*. He found that the fungus grew 25 per cent less on *P. infestans* culture filtrate resistant leaves than on control leaves. But no significant difference was observed in the number of sporangia produced between the control leaves and the leaves of the culture filtrate resistant plants.

Behnke (1980) selected dihaploid potato callus resistant to the culture filtrates of *Fusarium oxysporum*. The resistance was found to be carried to the regenerated plantlets also. However, further evaluation of resistance of there plantlets with the pathogen and inheritance of resistance was not reported.

Hartman and Secor (1985) challenged various tissues such as leaves, calluses and tubers *in vitro* with culture filtrates of *Verticillium dahliae* and *V. alboatrum* in potato. Leaves and calluses were found to react to the cultures or culture filtrate but the tubers were not.

Hess and Hess (1988) observed that resistant cultivars of potato were giving resistant calli while susceptible cultivars were giving susceptible calli when culture filtrates of *Verticillium dahliae* was used to screen potato cultivars.

The rate of *in vitro* multiplication of single node cuttings was used as a parameter for screening for resistance to *Verticillium dahliae* in potato in two selection cycles with different concentrations of *V. dahliae* culture filtrate (Sebastiani *et al.*, 1994). The resistant clone selected was evaluated by inoculating the fungus in the growth chamber and they reported that this selected clone was comparable to the resistant control cv. Kondor.

Tomato

Tomato plants obtained from calluses resistant to culture filtrate of *Alternaria solani* showed resistance to the disease (Shephered, 1986).

Similarly Illig and Dallacqua (1986) obtained resistant calli in tomato when screened for resistance to culture filtrate of *Phytophthora infestans*.

Toyoda *et al.* (1989a) used culture filtrate of a virulent strain of *Pseudomonas solanacearum* to select tomato resistant calluses. The plants regenerated from the resistant calluses expressed bacterial wilt resistance at the early infection stage to delay the growth of the inoculated bacteria. They observed complete resistance in self pollinated progeny of regenerants derived from non-selected callus tissues. These plants showed a high resistance when inoculated with that strain and were also resistant when planted in a field infested with a different strain of the pathogen.

Rice

The pathogenicity of culture filtrate from *Pyricularia oryzae* on rice was studied in detail by Zheng *et al.* (1985). Using the culture filtrate of four pathogenic races of *Pyricularia oryzae*. Li *et al.* (1986) screened both pollen calli and embryo calli and plantlets were regenerated from resistant calli. The R_1 generation of seedlings were inoculated with the corresponding race of the pathogen and the responses were evaluated. Twelve mutants with high resistance were selected out of the regenerated plants derived from 810 resistant calli of 10 cultivars. It was also found that the somaclones of resistant cultivars were still resistant.

Alfalfa

Hartman *et al.* (1984) observed increased resistance in alfalfa callus regenerants when culture filtrates of *Fusarium oxysporum* f. sp. *medicaginis* was used for screening the calli. Most regenerated plants obtained after a seven month selection cycle were chromosomally normal and resistant when inoculated with the pathogen. Calli derived from the somaclones were also filtrate resistant. Long term selection cycles yielded resistant plants with elevated ploidy levels. Preliminary results suggested the involvement of a dominant gene mutation. The resistance observed in the regenerants was found to be transmitted to the progeny. Similar resistance in callus regenerants when screened with culture filtrate of *F. oxysporum* f. sp. *medicaginis* was also reported by McCoy (1988).

Arcioni *et al.* (1987) also attempted *in vitro* selection of alfalfa calli for resistance to *Fusarium oxysporum* f. sp. *medicaginis*. The regeneration capacity of

the resistant calli was greatly reduced even though the lines originally selected were of high regeneration capability. Three plants out of the eight were resistant to the fungus and a high correlation between resistance to culture filtrate and *in vivo* resistance to the fungus was observed. However, inheritance of the trait was not tested. Binarova *et al.* (1990) also observed increased resistance in the regenerants to *F. oxysporum* f. sp. *medicaginis* when embryogenic suspension cells were selected using culture filtrate of the fungus.

Soyabean

The reaction of soyabean calli of resistant and susceptible genotypes to culture filtrate of *Phialophora gregata* was studied by Gray *et al.* (1986). Out of the seven genotypes studied calli from the five susceptible lines were sensitive to the culture filtrate and calli from two resistant genotypes showed resistance to the filtrate.

Song *et al.* (1994) regenerated soyabean plants resistant to host-specific pathotoxic culture filtrate of *Septoria glycines* by organogenesis from immature embryos of the cultivar BSR 201 and from mature seeds of three genotypes, BSR 201, Fayette and L 1615. The resistant plants obtained from immature embryos and mature seeds matured later than the parent.

Celery

Evenor *et al.* (1994) screened calluses derived from single cells of celery using culture filtrates of *Septoria apiicola*. Plants regenerated from resistant cells showed different degrees of tolerance to *S. apiicola* in green house tests and selfing the selected plants yielded tolerant progenies.

Rape

Sacristan (1982, 1985) challenged *Brassica napus* suspension cells and embryo cultures with culture filtrates of *Phoma lingam* in the ratio 1:1 added to the tissue culture medium. The resistance obtained in culture was found in the regenerants and transmitted to the progeny also. Two plants out of 29 showed high degree of resistance and did not show any symptoms several months after inoculation.

Partially purified culture filtrate of *Alternaria brassicicola* when used to select embryoids, increased resistance was observed in rape (Mac Donald and Ingram, 1985, 1986).

Beans

Bajaj and Saettler (1968, 1970) reported inhibition of growth of bean callus at 5 and 10 per cent culture filtrate concentrations of *Pseudomonas phasiolicola*. Hartman *et al.* (1985) opined that callus screening system could identify bean cultivars resistant to halo blight since resistant calli were giving resistant regenerants and susceptible calli susceptible regenerants.

Onion

The calli induced from resistant lines of onion to *Pyrenochaeta terrestris* when grown in the medium containing culture filtrate, there was no growth inhibition and reduction in weight. But the calli of susceptible lines had a more pronounced reduction in weight and exhibited pigmented and necrotic areas in the tissue (Gourd and Phillips, 1986).

Barley and wheat

Chawla and Wenzel (1987b) used two selection methods to screen barley and wheat genotypes for resistance to purified culture filtrate produced by the fungus *Helminthosporium sativum* - a continuous method in which four cycles of selection were performed one after another on toxic medium and discontinuous method in which a pause on nontoxic medium was given after the second or third cycle of selection. The latter was found superior as it allowed the calli to regain their regeneration ability. Toxin tolerant callus lines of barley were characterised by protein isozymes and zymograms showed one more isozyme in the unselected sensitive callus. Majority of the regenerants when tested against the pathogen were found to be less sensitive. This was further confirmed by Chawla and Kole (1990).

Ahmed *et al.* (1991) employed double layer culture technique to select spring and winter wheat calli resistant to *Fusarium graminearum* and *F. culmorum*. Potato dextrose agar medium in vials was inoculated with mycelia of the fungus. After one week, fungal cells were killed by autoclaving and the agar medium containing the thermostable toxic metabolites was overlayered with MS callus growing medium. Calli were inoculated on the upper medium and incubated for 4-5 weeks. From the surviving calli plants were regenerated. R_2 seedling populations from self fertilized R_1 plants of four varieties were tested for *Fusarium* resistance by artificial inoculation in the green house and three per cent of the regenerated R_2 plants have been found to be more resistant than the original cultivars.

Ginger

Kulkarni *et al.* (1984) induced calli from healthy sprouting buds of ginger cultivars Mahim and Poonam local and the suspensions made from calli were grown in liquid MS medium containing 10 and 20 per cent culture filtrates (CF) of the fungus *Pythium aphanidermatum*. Fifty per cent inhibition of colony formation was found in media containing 10 per cent culture filtrate and total inhibition was found in 20 per cent CF. The morphogenic potential of the callus cultures decreased with every successive subculture on media with 20 per cent CF. Only three rhizome samples out of 114 of cv. Mahim were tolerant to *Pythium* where as all the rhizome samples of cv. Poonam local were susceptible to the fungus. They further observed that there was no yield reduction in the screened and unscreened plants.

Musk melon

Meghegneau and Branchard (1991) studied the effects of fungal culture filtrates on tissue from susceptible and resistant genotypes of musk melon to *Fusarium oxysporum* f. sp. *melonis*. (Fom) Cotyledon fragments derived from susceptible and resistant genotypes were sensitive to the culture filtrates of pathogenic and non pathogenic isolates of Fom. Based on the work, they concluded that *in vitro* bioassay could not be used for evaluating sources of resistance in musk melon to Fom or for assessing specific pathogenicity of the fungus isolates.

Citrus

Vardi *et al.* (1986) subjected nucellar calli from four citrus cultivars with known resistance to *Phytophthora citrophthora* to culture filtrate (CF) of the fungus.

They found that sensitivity of the four calli to culture filtrate of the fungus was in reverse order to what is known on the susceptibility of the cultivars *in vivo*. Protoplasts derived from calli selected for tolerance to culture filtrate showed a higher plating efficiency with increasing concentration of CF in the medium. TLC and GLC determinations showed the presence of indole acetic acid in the culture filtrate. They concluded that culture filtrate of *Phytophthora citrophthora* could not be used as a selection tool *in vitro*.

Eggplant

Rotino *et al.* (1987, 1990) reported increased resistance to *Verticillium dahliae* in regenerants when suspension cells were screened using culture filtrate of the pathogen. Progeny testing and inheritance of the trait was not reported.

Hop

Reduced symptom expression to *Verticillium albo atrum* was reported in Hop by Connell and Heale (1987) when calluses were selected using culture filtrate of the pathogen.

Peach

Hammerschlag (1988) reported screening of peach calluses using fractionated culture filtrate of *Xanthomonas campestris* pv. *pruni*. He observed increased resistance in the regenerants, the inheritance of resistance however was not studied.

Tobacco

Selvapandiyan *et al.* (1988) screened suspension cells of tobacco using culture filtrate of *Fusarium oxysporum* f. sp. *nicotianae*. Enhanced tolerance in the regenerants was reported.

Red gram

Production of disease resistant plants in red gram through cell and tissue culture was reported by Rao and Basavaraj (1995). Fusarium culture filtrate (FCF) was employed for callus screening. Fusarium culture filtrate at 30 and 40 per cent concentration drastically reduced the growth of callus tissue and 40 per cent concentration was used to select resistant callus lines. The regeneration from selected cell line was achieved but with very low frequency.

2.1.2.2 Purified toxins/toxin analogues as screening agent

Tobacco

Carlson (1973) first demonstrated that plant cells and protoplasts could be selected *in vitro* for resistance to diseases and plants with an altered response to infection by the pathogen could be regenerated from these cultured cells. He attempted selection for *Pseudomonas syringae* pv. *tabaci* in tobacco using methionine sulfoximine, an analogue of the toxin of the pathogen. Calli derived from mutagenised haploid tobacco cells when screened, number of variants had been recovered. Three cultures with stable resistance were diploidised, regenerated into plants, tested for resistance to both the screening agent and pathogen. Plants from two of these lines showed enhanced resistance to *P. tabaci* though the intensity of infection was more compared to the naturally resistant variety.

The toxic fraction from culture filtrate of *Alternaria alternata* was used by Thanutong *et al.* (1983) for screening tobacco and he observed resistance both in culture and regenerated plantlets. The increased resistance to the pathogen was transmitted to approximately 10-20 per cent of the regenerated progeny.

Kumashiro (1983) selected tobacco cell cultures resistant to tenuazenic acid, a non specific toxin produced by *Alternaria alternata*. Plants regenerated from resistant cells were susceptible to the toxin. When cells were isolated from these plants and put back in culture they again expressed toxin resistance. The resistance selected in cell culture was not expressed at the whole plant level.

Maize

Gengenbach and Green (1975) developed maize lines resistant to *Helminthosporium maydis* ract T by conducting toxin selection with callus cultures of Cms-T and N versions of A 619 maize after four subculture cycles on toxin. The regenerants were resistant both to the pathogen and the toxin. The resistance was associated with a reversion to male fertility, both characters showed maternal inheritance. This finding was again confirmed by the work of Brettel *et al.* (1980).

Gengenbach *et al.* (1981) observed that Cms-T male sterility and sensitivity to *Dreschlera maydis* T-toxin had been ascribed to alterations in the mitochondrial genome.

Sugarcane

Eye spot toxin tolerance in sugarcane was studied in detail by Heinz *et al.* (1977) and Larkin and Scowcroft (1981, 1983).

A rapid and highly repeatable bioassay based on increase in conductivity of tissue leachates was developed by Larkin and Scowcroft (1983). A total of 480 somaclones from the cultivar Q 101 which included plants derived from cultures with and without toxin selection were screened for tolerance to toxin and a very high frequency of toxin tolerant variants was found. Eighty five somaclones when analysed for stability to increased toxin tolerance it was observed that 22 per cent were more tolerant, 38 per cent were more susceptible. The tolerant variants when passed through a second tissue culture cycle it was found that 40 per cent of plants had a similar tolerance to primary somaclone, 22 per cent were more tolerant and 38 per cent were more susceptible.

Rice

In vitro screening of rice germplasm for resistance to brown spot disease was done by Ling *et al.* (1985) and they first reported about the disease resistant mutation obtained successfully in rice by tissue culture and *in vitro* screening with phytotoxin. Two R_1 IR 8 plants derived from somatic cells cultured in 25 per cent *Helminthosporium oryzae* toxin medium and one IR 54 plant derived from toxin free medium was found to have mutated resistance to brown spot disease. In the next generation the segregation for resistance and susceptibility was observed as a dominant mutation.

Further studies on selection of brown spot resistant rice plants from *H. oryzae* toxin resistant calluses were done by Vidyasekharan *et al.* (1990). They observed that *H. oryzae* toxin induced electrolyte leakage from rice callus tissues and caused browning and death of callus tissues. A virulent isolate of the pathogen

invaded and colonised the calli rapidly while a less virulent and nonpathogenic isolate colonised the calli slightly and addition of the toxin to the calli permitted colonisation of calluses by the nonpathogenic isolate. Four toxin resistant calluses were selected after screening by toxin shaking and plants regenerated from two of these resistant calluses showed resistance to the pathogen. This resistance was heritable and stability of resistance was observed in the R_1, R_2 and R_3 generations.

A detailed study on the cellular resistance in rice to cercosporin was reported by Batchvarova *et al.* (1992). Four rice cultivars differing in resistance to *Cercospora oryzae* were examined for resistance to purified cercosperin. The cultivars were red rice (most resistant to pathovars of *C. oryzae*) Lemont and Leah (intermediate resistance) and Labelle (most susceptible). Resistance to toxin was assessed in seedlings (inhibition of growth), leaves (chlorosis and necrosis), callus (inhibition of growth and hydrolysis of fluorescein di acetate) and cell suspensions (ion leakage). Toxicity of cercosporin depended on illumination. Labelle proved to be the most susceptible cultivar in each test, whereas red rice showed extreme resistance to the toxin and grew in the presence of cercosporin concentrations that were toxic to labelle. Treating cell suspensions of red rice with norflurazon abolished the cercosporin resistance in this cultivar and carotenoids were implicated in the resistance mechanism.

Tomato

Effects of *Alternaria alternata* f. sp. *lycopersici* toxin at different levels of tomato plant cell development was studied by Witsenboer *et al.* (1988). The effect of toxin on leaves, leaf discs, roots, calli, suspension cells, mini calli and protoplasts of susceptible and resistant genotypes were compared. In leaves of

susceptible genotypes toxins cause severe necrosis while in leaves of resistant genotypes necrosis was never observed. Toxins inhibited shoot induction from leaf discs, root growth, growth of calli, suspension cells and protoplasts. Differences in sensitivity to AAL toxins between susceptible and resistant genotypes were observed in leaves and roots but were not observed during shoot induction, in calli, in suspension cells and protoplast.

Shahin and Spivey (1986) reported the possibility to isolate *Fusarium oxysporium* f. sp. *lycopersici* race 2 resistant tomato lines from both fusaric acid (non-specific toxin) challenged and non-challenged protoplasts. Protoplasts were isolated from cotyledonary tissues of tomato cv. 'UC-82' which was susceptible to *F. oxysporum* race 2. They got resistant plants both from challenged and non challenged protoplasts in the same intensity. In both cases a single dominant type resistance was recovered and transmitted through several sexual generations.

Potato

Protoclones of potato (cv. Russet Burbank) regenerated from cultured protoplasts were tested for disease response by inoculating the leaves with partially purified toxins of *Alternaria solani* (Matern *et al.*, 1978). Five hundred protoplast derived plants were tested and four types of reaction were observed (1) highly sensitive (2) sensitive (3) intermediate and (4) insensitive. In highly sensitive plants the whole leaf blade turned yellow and died, where as the other classes differed in the size of the lesions. The insensitive plants retained their characteristics of disease response over successive generations.

Wenzel *et al.* (1987) extracted purified toxin of *Phytophthora infestans* by ultrafiltration. The effectiveness of the toxin was tested at different concentrations by measuring the increase in fresh weight of shoot-tip cultures. The sensitive calli to toxin turned black and they could regenerate plants from resistant calli.

The protoplast population of four different haploid clones of potato were screened on defined culture media containing exotoxin(s) produced by *Fusarium sulphureum* and *F. solani* var. *coeruleum*. One thousand and five hundred protoclones were regenerated from resistant protoplasts of the four clones (Wenzel *et al.*, 1987).

Progeny testing of *in vitro* selected regenerants of potato using toxic fractions of *Phytophthora* and *Fusarium* was reported by Wenzel and Foroughi-Wehr (1990). They found that progenies did not differ significantly in their level of susceptibility from the susceptible starting material. The concluded that this might be due to an inadequate selective agent used *in vitro*, to a lack of genetic differences, or to a test procedure that was not sensitive enough to detect slight alterations in the level of susceptibility.

Oats

Rines and Luke (1985) observed toxin resistance in 74 out of 1216 oat calli grown in *Helminthosporium victoriae* toxin medium. The plants regenerated from toxin calli showed resistance to the disease.

Lettuce

Engler and Grogan (1982) used oxalic acid as screening agent for *Sclerotinia* spp. in lettuce. The resistance observed in culture was not found in the regenerated plantlets.

Bean

The phaseolo toxin produced by *Pseudomonas syringae* pv. *phaseolicola* when used for screening bean calluses, the resistant calluses was found to give resistant regenerants (Gantotti *et al.*, 1985).

Citrus

Nadel and Roy (1987) selected *Citrus limon* cell culture variants resistant to the malsecco toxin produced by *Phoma tracheiphila* for six consecutive subcultures. Viability of cell lines was monitored by the use of fluorescein diacetate. The stability of the resistance was examined after growth on nonselective medium for three subcultures. The variant line var. 1.117 showed stable resistance and maintained their embryogenic capacity. Callus induced from the somatic embryos also displayed resistance to the toxin.

Leucerne

Resistance to *Verticillium* wilt in Leucerne was studied by Latunde-Dada and Lucas (1988) by employing a low molecular weight toxic fraction from culture filtrate of *Verticillium albo-atrum* to the callus medium prior to regeneration. Toxin treatment reduced regeneration potential of the calli and there were no resistant plants regenerated from calli selected even at low toxin concentrations. They opined

that the inhibitory effects of the toxin(s) on regenerative capacity, coupled with the shift towards resistance in unselected control regenerants might have masked any beneficial effects of the selection regime.

Poplar

Antonetti and Pinon (1993) observed that calli of Leuce poplars that had survived the exposure to increasing concentrations of toxins from *Hypoxylon mammatum* gave rise to toxin tolerant plants. Flow cytometry to measure the DNA content of nuclei showed that regenerants were found to be tetraploids.

Muskmelon

Response of leaf spot sensitive and tolerant muskmelon cells to phytotoxin roridin E was studied by Healey *et al.* (1994). The response of phytotoxin produced by *Myrothecium roridum* was monitored by measuring changes in the fluorescence of cells stained with merocyanine 540, an optical probe for changes in transmembrane electric potential (PD). The fluorescence emission from leaf spot sensitive and tolerant muskmelon mesophyll cells was not affected by roridin E. However, suspension cells from only the leaf spot sensitive cultivar "Iroquois" exhibited a significant increase in fluorescence after 5 minutes of exposure to 10 μM roridin E. They concluded that roridin E was a good *in vitro* selective agent for muskmelon suspension cells and that fluorescence measurements might be useful in screening muskmelon germplasm for resistance to *M. roridum*.

Daub (1986) and van den Bulk (1991) listed out some problems encountered when selecting with phytotoxins. According to Daub (1986) lack of sufficiently characterised toxins that play a role in important diseases, differential

expression of toxin resistance at single cell and whole plant levels and the possibility that the levels of resistance desired cannot be obtained owing to the mode of action of the toxin involved were the major technical problems.

There are many diseases in which no toxin has been identified and many diseases in which toxins have been identified but have not been shown to play a role in disease (Scheffer, 1983). The role of phytotoxic compounds isolated from culture filtrates of pathogenic fungi and bacteria in pathogenesis has also not yet been elucidated (Goodman *et al.*, 1986).

Host-specific toxins have been considered as primary determinants of the disease and hence the calluses resistant to host-specific toxins may give rise to plants with high degree of resistance (Vidyasekharan, 1990). Host-specific toxins, with a primary role in pathogenesis have been used successfully in selection experiments, (Gengenbach *et al.*, 1977; Larkin and Scowcroft, 1983; Rines and Luke, 1985; Chawla and Wenzel, 1987b) and excellent correlations have been demonstrated between resistance to the pathogen at the plant level and insensitivity of the toxin at the cellular level.

Non-specific toxins are usually regarded as the secondary determinants of the disease or virulence factors, ie. compounds which increase the extent of the disease symptoms but are not involved in the primary interaction that determines compatibility. Consequently, plant resistant to these toxins probably do not show complete resistance to the pathogen (van den Bulk, 1991). However, increased resistance to pathogens using non-specific toxins as screening agent was reported by Thanutong *et al.* (1983); Hammerschlag (1986); Chawla and Wenzel (1987a) and Pauly *et al.* (1987).

The reliability of selections using non-specific toxin have often been questioned. Lack of correlation between *in vitro* response to toxin and response of the whole plant to infection has been noticed in several systems in which non-specific toxins are involved (Branchard, 1982; Kumashiro (1983); Daub, 1984; Vardi *et al.* 1986). Still, selecting for resistance to non-specific toxins is important, since resistance to these toxins does not occur naturally and one must generate sources of resistance not currently available.

Culture filtrates are not the best approach to select for resistance since there are chances for selecting for resistance to non-specific substances found in the filtrates (Daub, 1986). When using crude culture filtrates, it is important to generate as many resistant lines as possible. Many of these lines might have been selected for resistance to components other than the putative toxin, but if enough are generated, some may carry the desired resistance. Culture filtrates have to be used with care, as shown by Vardi *et al.* (1986) who investigated suitability of *Phytophthora citrophthora* culture filtrate as an *in vitro* selection tool. Although the culture filtrate contained toxic components and showed activity at the cellular level, their results indicated that effects on cells may be due to interaction with secondary metabolites, possibly auxin like substances, not involved in the disease development.

The mode of action of toxins and interaction of toxins with different types of tissues may affect selection *in vitro* (van den Bulk, 1991). The importance of the mode of action and properties of toxins was also demonstrated by the work of Daub (1984) who was unsuccessful to select cells of tobacco and sugarbeet resistant to the non-specific toxin cercosporin.

Inspite of the large number of successes listed in *in vitro* screening using phytotoxins, only in about 50 per cent of the investigations, inheritance studies were carried out (van der Bulk, 1991). Very few of these studies have been taken beyond the laboratory and green house and no varieties generated in this way have been released (Daub, 1986). For studies that have taken to the field, the results have been disappointing or difficult to interpret. Potato lines selected for resistance to culture filtrates of *Phytophthora infestans* have been field tested but losses due to other diseases have prevented accurate assessment of their resistance to late blight (Wenzel, 1985). Four lines of spring rape selected for resistance to culture filtrates of *Phoma lingam* have shown greater resistance compared to the susceptible parent but are not as resistant as some varieties currently available (Sacristan, 1982 and Daub, 1986). The progenies of several regenerants of barley, wheat, potato that were selected for resistance to toxic fractions of *Helminthosporium, Fusarium* and *Phytophthora* did not differ significantly in their level of susceptibility from the susceptible starting material (Wenzel and Foroughi-Wehr, 1990). In contrast, field evaluation of alfalfa selected for resistance to culture filtrates of *F. oxysporum* f. sp. *medigaginis* (Hartman, 1983; Hartman *et al.*, 1984) have been encouraging.

2.2 Production of calliclones without *in vitro* screening and screening the regenerants at the whole plant level

Isolation of disease resistant variants without applying any selection pressure *in vitro* is another approach. This system utilises the high frequency of stable heritable variation generated in tissue culture cycle and screening for disease resistance is done at the whole plant level using the conventional methods of disease resistance screening. Somaclonal variation has been extensively exploited for isolating disease tolerant/resistant types in crops like sugarcane, potato and tomato.

Sugarcane

Krishnamurthi and Tslaskal (1974) and Heinz *et al.* (1977) regenerated plantlets from callus cultures of shoot meristem. Plants regenerated were found to be resistant to Fiji virus. The increased resistance was found to be transmitted to the vegetative propagules also. Krishnamurthi (1974) and Heinz *et al.* (1977) reported increased resistance to *Sclerospora sacchari* in callus regenerants of sugarcane. Increased resistance to *Helminthosporium sacchari* in regenerants from suspension cultures was reported by Heinz *et al.* (1977). Larkin and Scowcroft (1983) also reported resistance to *Helminthosporium sacchari* in sugarcane calliclones. Liu and Chen (1978) and Liu (1981) observed increased resistance to *Ustilago scitaminea* in regenerants from calli. Field resistance to *Puccinia melanocephala* in callus regenerants was reported by Sreenivasan *et al.* (1987).

Tomato

Resistance to tobacco mosaic virus, Fusarium wilt and bacterial wilt has been reported in somaclones of tomato by several workers.

Barden *et al.* (1986); Smith and Murakishi (1987) reported resistance to TMV in tissue culture regenerants. The resistance was found to be transmitted to the progenies. Calliclones showing monogenic resistance to *Fusarium oxysporum* f. sp. *lycopersici* race 2 was observed by Miller *et al.* (1985) and Evans (1987). Shahin and Spivey (1986) regenerated protoclones from cotyledonary tissue of 'UC-82' which was susceptible to *Fusarium oxysporum* f. sp. *lycopercisi* race 2. Analysis of the R_2 progenies showed that *Fusarium* resistant plants were either homozygous or heterozygous dominant for the gene confirming the resistance.

Suppressed bacterial growth was observed in tomato calliclones when screening was done against *Pseudomonas solanacearum* (Toyoda *et al.*, 1989b).

Potato

Plants regenerated from mesophyll protoplast of potato showed resistance to *Alternaria solani*. The resistance observed in protoclones found to be transmitted to the vegetatively propagated progeny (Matern *et al.*, 1978). Shepard *et al.* (1980) reported resistance to *Phytophthora infestans* in protoclones of potato. Meulemans and Fouarge (1986) also reported increased resistance to *Phytophthora infestans* in protoclones, calliclones and explant derived plantlets of potato. Evans *et al.* (1986) evaluated somaclonal variation in explant derived potato clones over three tuber generations. They observed that in all the years the population of clones had fewer scab (*Streptomyces scabies*) lesions than the controls. Increased field resistance to *Streptomyces scabies* in protoclones was also reported by Thomson *et al.* (1986).

Jellis *et al.* (1984) reported resistance to leaf roll virus in somaclones of potato. Field resistance to potato virus x and potato virus y was observed in calli clones and explant derived plantlets by Cassels *et al.* (1986, 1987). Increased field resistance to potato virus y and potato leaf roll virus was reported by Thomson *et al.* (1986).

Rice

In rice Ling *et al.* (1985) identified one plant among the regenerants derived by somatic cell culture of variety IR 54 which was resistant to the brown spot disease caused by *Helminthosporium oryzae*. The progeny of this plant

segregated for resistance and susceptibility in the ratio 5:4. The resistance was suggested to be conferred by a dominant mutation.

Tobacco

Calliclones of tobacco were found to be tolerant to *Peronospora tabacina* and tobacco mosaic virus. The resistance was found to be transmitted to the progeny (Zagorska and Atanassov, 1985).

Daub and Jenns (1989) analysed a total of 854 protoclones of two flue-cured tobacco cultivars and their progeny in green house and field for yield, leaf chemistry and resistance to black shank, bacterial wilt, tobacco mosaic virus and root knot (*Meloidogyne incognita*) nematode. Progeny of the somaclones had normal phenotype and did not differ significantly from the parent cultivars in yield and leaf chemistry. Significant variation was found in resistance to black shank and bacterial wilt, two diseases for which the parental cultivars have low levels of resistance. No somaclones were identified with resistance to TMV and *M. incognita*. They concluded that genetic variation occurred in the somaclones, that the magnitude of variation was slight and the variation depended on both the genotype of the parent cultivar and the trait.

Alfalfa

Resistance to *Verticillium albo atrum* in protoclones derived from mesophyll protoplasts was reported by Latunde-Dada and Lucas (1983). Hartman (1983) and Hartman *et al.* (1984) observed resistance to *Fusarium oxysporum* f. sp. *medicaginis* in calliclones. Binarova *et al.* (1990) also observed resistance to *Fusarium oxysporum* f. sp. *medicaginis* and *Fusarium solani* in regenerants derived

from embryogenic cell suspensions. However, Arcioni *et al.* (1987) could not isolate any resistant plants to *F. oxysporum* f. sp. *medicaginis* from regenerants of leaf calli.

Maize

Brettel *et al.* (1980) and Umbeck and Gengenbach (1983) observed resistance to *Helminthosporium maydis* race T in clones derived from calli of mature embryos.

Rape

Plantlets derived from calluses and embryogenic cell suspension when screened against *Phoma lingan*, increased tolerance which was found to be transmitted to the progeny was observed by Sacristan (1982, 1985). Increased resistance to *Alternaria brassicicola* in plantlets regenerated from secondary embryoids was reported by MacDonald and Ingram (1985, 1986). However, the inheritance of the trait was not tested.

Celery

Plants regenerated from cell suspension cultures were found to give highly resistant plants to *Cercospora apii, Septoria apiicola, Pseudomonas cichorii, Fusarium oxysporum* f.sp. *apii* race 2 (Wright and Lacy, 1988). Resistance to *Fusarium oxysporum* f.sp. *apii* in plants derived from callus and embryogenic suspension cultures was also reported by Ireland and Lacy (1987), Heath-Pagliuso *et al.* (1988), Heath-Pagliuso and Rappaport (1990).

Lettuce

Plants regenerated from cotyledon calli were screened for reaction to lettuce mosaic virus (LMV) and downy mildew (*Bremia lactucae*). Increased resistance to lettuce mosaic virus and reduced and delayed sporulation for *Bremia lactucae* were observed in the clones (Brown *et al.*, 1986). All the three cultivars studied viz. Salad Bowl, Lobjoits Cos and Pennlake exhibited somaclonal variation.

Apple

Rosati *et al.* (1990) observed increased resistance to toxic culture filtrate of *Phytophthora cactorum* in tissue culture regenerants.

Banana

Hwang and Ko (1988) observed increased resistance to *Fusarium oxysporum* f.sp. *cubense* in meristem culture derived plantlets.

Barley

Increased field resistance to *Rhynchosporium secalis* in calliclones was observed by Pickering (1989). Increased resistance to *Helminthosporium sativum* was reported by Chawla and Wenzel (1987b) in barley somaclones.

Wheat

Chawla and Wenzel (1987b) observed resistance in wheat somaclones to *Helminthosporium sativum*.

Strawberry

Toyoda *et al.* (1991) selected two resistant lines of strawberry to *Fusarium oxysporum* f. sp. *fragariae* out of a total of 1,225 somaclones derived from leaf calli. The selection of disease resistant strawberry was attempted by transplanting regenerated plants to a pathogen infested soil.

Peach

In peach calliclones increased resistance to *Xanthomonas campestns* pv. *pruni* and *Pseudomonas syringae* pv. *syringae* was reported by Hammerschlag (1990a, b) and Hammerschlag and Ognjanov (1990). The resistance observed in calliclones was found to be transmitted to the vegetative propagules also.

Poplar

Somaclonal variation in eastern cotton wood for race specific partial resistance to leaf rust disease was reported by Prakash and Thielges (1989). Forty somaclones derived from leaf calli were tested for their reaction to two races of *Melampsora medusae* using the leaf disc assay. Most somaclones had leaf rust reactions similar to those of the parent but some were more susceptible.

Ostry and Skilling (1988) reported resistance to *Septoria musiva* in poplar calliclones. In a more detailed study Ostry *et al.* (1994) reported that explant source and culture method influenced the recovery of variant plants in hybrid populus. The regenerants expressed somatic variation in disease resistance in different frequencies except for plants regenerated from hard wood cuttings. Of the 1065 plants regenerated from all explant sources 10.2 per cent were highly resistant.

The greater frequency of variants was obtained from stem callus cultures followed by somatic embryos and the lowest from plants regenerated from roots. Plants from stem callus cultures were more resistant than plants obtained from any other explant sources.

Water cress

Arnold *et al.* (1995) reported somaclonal variation in water cress (*Rorippa nasturtium-aquaticum*) for resistance to crook root disease. The regenerated plants from petiole calluses were screened for resistance to crook root disease using a laboratory screening test. Significant somaclonal variation between the control and some of the somaclones was observed in the screening test. Although none showed complete resistance, five somaclones showed reduced level of disease symptoms compared to the control.

There are obvious advantages to relying on the high frequencies of variation obtained in culture to isolate variants in the absence of selection (Daub, 1986). Accurate and workable selection schemes using either pathogen or pathogen toxins are difficult to devise which needs considerable time and effort and must be made on the basis of host-pathogen interaction. Since the selection step is eliminated in this system there is no need to characterise new toxins or devise methods by which uniform inoculum is given to cultures. Further, the desired traits are screened in the regenerated plant. So the problem of differential gene expression at the single cell and whole plant level can be avoided. If selection schemes are not involved it is necessary only to devise an efficient regeneration system and to use conventional methods of disease screening to select resistant plants.

van den Bulk (1991) stressed the advantage of selection of dominant and homozygous recessive trait in the regenerants. If the regenerants are heterozygous for a desired character, recessive traits can be selected in the progenies of the regenerants there by avoiding epigenetic variation.

This system has got several disadvantageous also (Daub, 1986). It is not always possible to obtain the desined lines even if very large numbers were screened. Wenzel (1980) starting from protoplasts of dihaploid potato could not find an useful variant among more than 3000 regenerated clones.

Somaclonal variation results in changes in multiple traits. Bidney and Shepard (1981) analysed 26 different traits in 63 potato clones and found that one clone varied in as many as 17 traits and only 3 clones varied in a single trait. Thus lines derived from culture may have the desired traits but may also have been altered in other undesirable ways.

A great deal of effort is required to regenerate plants in large numbers and screening the somaclones accurately if selection step is not applied in the early stage.

To date, the selection of somaclonal variants with disease resistance has only resulted in the release of only a few cultivars.

The varieties released till now were derived from screening the regenerants at the whole plant level. A sugarcane cultivar "Oono" which showed resistance to Fiji disease (Daub, 1986), a tomato variety DNAP-17 with mono genic Fusarium wilt race 2 resistance (Evans, 1989) and a celery line 'UC-T3 somaclone' with resistance to *Fusarium oxysporum* f. sp. *apii* were released. However, in

general resistant selection have not advanced beyond the laboratory or green house phase either due to the occurrence of unwanted genetic variation or because the resistance trait obtained was not a novel one. van den Bulk (1991) concluded that these novel techniques may be applied when resistant material is not available or when the incorporation of known resistance genes in existing varieties is difficult.

2.3 *In vitro* induction of mutation using gamma irradiation

In recent years there has been a major thrust in the application of mutagens especially physical mutagens like gamma rays in plant tissue culture. However, literature is scanty about the successful selection of disease resistant mutants after *in vitro* application of mutagens. Most successes have been achieved without an additional mutagenic treatment which suggests that sufficient variation has been generated by the cell.

2.3.1 Effect of gamma irradiation on callus growth and differentiation

Beans

The effect of gamma irradiation on bean tissue cultures was studied by Bajaj *et al.* (1968). Three day old suspension cultures containing free cells, aggregates of cells and small callus masses were subjected to gamma radiations at various doses (500 R, 1, 2, 5, 10, 20, 30, 40 and 50 kR @ 200 R/minute) and grown for four weeks in diffused light. At 500 R there was a slight stimulation of growth as assayed by tissue volume fresh weight, dry weight and mitotic index. However, from 1-10 kR there was a gradual decrease in growth. The cells exhibited a wide variety of shapes and sizes, mitotic inhibition, increased polyploidy, degeneration of cytoplasm and reduced plating efficiency. At 20 and 30 kR, growth

was drastically reduced and inhibited. Severe killing and cessation of growth occurred at 40 kR. The colour of the callus continued to darken with increased dosimetry.

Bajaj (1970) further reported about the RNA, protein and nitrogen contents of bean callus cultures subjected to gamma irradiation. He found that with increase in the doses of gamma irradiation, RNA and soluble protein continued to decrease. At lower doses (0.5 and 1 kR) there was no significant difference in total nitrogen between the control and irradiated cultures. However, from 2 kR upwards there was a gradual increase in total nitrogen in terms of μg/mg dry weight of the irradiated callus. These results demonstrate that there is a direct correlation between growth, RNA and protein levels. Gamma irradiation in general caused inhibition of tissue culture growth along with failure of synthesis of RNA and subsequently protein.

Bajaj *et al.* (1970) compared the effect of ^{60}Co gamma radiation on seeds, seedlings and callus tissue culture of *Phaseolus vulgaris* L. var. Manitou. They observed striking difference in the radio sensitivity of seeds, seedlings and callus tissue, the young seedlings were most radio sensitive followed by seeds and callus tissue was most tolerant.

Tobacco

A comparative study of the effects of gamma irradiation was carried out on young seedlings of tobacco in culture and callus tissue grown in suspension culture by Venketeswaran and Partanen (1966). A striking difference in radio sensitivity was observed between the seedlings and callus tissue. Low doses of 650

embryoid formation. With low kinetin and IAA, irradiation depressed callus growth and stimulated embryoid differentiation.

Mustard

George and Rao (1980) reported *in vitro* regeneration of mustard plants from irradiated, non-irradiated and mutagen treated seeds. Gamma rays in doses above 2 kR suppressed shoot regeneration from cotyledon explants of the var. Rai-5 but stimulated callus growth. Inhibition of rooting was evident at 15 kR and above this dose roots were rarely initiated. All the shoots produced from cotyledon cultures were small, poorly developed and only few plants could be produced.

Rice

Direct and indirect effects of gamma rays on stimulation of morphogenesis in long term tissue culture of rice was reported by Sharma *et al.* (1983). Eight month old calluses which had lost the capacity to differentiate shoots were exposed to 0.5, 1 and 2 kR doses of gamma rays in a chamber having an intensity of 23.3 kR/hour. Irradiating the calluses with 1 kR dose had a pronounced stimulating effect on plant regeneration (80%) than irradiating the medium (66%). Irradiation of both the medium and calluses resulted in regeneration of 33 per cent calli only. Exposure of 0.5 kR did not induce regeneration and 2.0 kR dose caused blackening of the calli without further growth.

Datura

The effect of gamma irradiation on callus growth and shoot regeneration in *Datura innoxia* was reported by Jain *et al.* (1984). Explants from anther derived

haploid plantlets were used for initiating the calli. Growth of callus cultures was stimulated at 0.2 kR dose of gamma irradiation but it decreased as radiation dose increased. Cultures exposed to 5 kR dose turned brown indicating a general inhibition of callus growth. Shoot regeneration was stimulated both at 0.2 as well as 1 kR radiation dose while no shoot regeneration was observed in 5 kR irradiated cultures. Differentiation in irradiated cultures occurred 10-12 days earlier than in unirradiated cultures.

Maize

In maize callus also both growth and plant regeneration capacity decreased with increasing levels of gamma irradiation (Moustafa *et al.* 1989). The plant regeneration capacity was more sensitive than the callus growth. The decrease in plant regeneration capacity was correlated with a change in tissue composition of the treated callus from a hard yellow and opaque tissue to a soft greyish yellow and translucent tissue. This change was quantified by measuring the reduction of MnO_4 to MnO_2 (PR assay) by the callus. They further suggested that PR assay could be used for predicting the actual plant regeneration capacity of maize callus.

Chillies

The induction of multiple shoots from cotyledon explants of *Capsicum annuum* cultivar CA 960 by gamma irradiation was reported by Subhash and Prolaram (1987). The lower dose of 0.1 kR of gamma irradiation proved stimulatory in inducing multiple shoots while doses of 0.5, 1.0, 2.5 and 5 kR caused total suppression of shoot bud formation.

Banana

Difference in radio sensitivity and post radiation recovery among different *Musa* clones was reported by Novak *et al.* (1990). They subjected the shoot apices with two pairs of leaf primordia from *in vitro* cultured shoots of seven clones of dessert banana, plantain and bluggoe cooking banana to gamma irradiation of 15, 30, 45 and 60 Gy at a dose rate of 8 Gy min^{-1}.

Gooseberry

Aseptically cultured shoots of chinese gooseberry exhibited growth disorder and morphological aberrations when subjected to gamma irradiation (Shan Shen *et al.*, 1990). The LD 50 worked out was 80-90 Gy and 50-60 Gy respectively for cv. Hayward and clone 4. All petiole explants irradiated with gamma ray could form calli but the rate of differentiation of adventitious shoots decreased when subjected to gamma irradiation and the decrease was dependent on dose. Sensitivity of the shoot or petiole explants to gamma irradiation varied with species.

2.3.2 *In vitro* induction of mutation and the recovery of disease resistant mutants

Carlson (1973) reported resistance to wild fire disease of tobacco caused by *Pseudomonas syringae* pv. *tabaci*, when calli derived from mutagenised haploid tobacco cells were screened using methionine sulfoximine, an analogue of the tab toxin. He used 0.25 per cent ethyl methane sulfonate for treating cells and protoplasts.

Behnke (1979) subjected potato calli grown in media containing culture filtrate of *Phytophthora infestans* to X-rays 2000 R. He observed that the mutation

frequency of the irradiated calli was not much higher than that of the non-irradiated calli.

Sacristan (1982) treated cell suspension and embryo cultures of *Brassica napus* with chemical mutagens to increase the variation in culture so that plants with an enhanced tolerance to black leg disease (*Phoma lingam*) could be selected.

Murakishi and Carlson (1982) reported only limited resistance to TM-Flavum in tobacco after gamma irradiation of the plant material.

Daub (1986) could not isolate cercosporin resistant cells from protoplasts isolated from haploid tobacco plants mutagenised by irradiation with 600 rads of acute gamma radiation.

Irradiation of *in vitro* explants and subsequent adventitious regeneration has been tested by Pinet-Leblay *et al.* (1992) in four commercially important varieties of pear (*Pyrus communis*) with the aim to create mutants with a reduced susceptibility to fire blight (*Erwinia amylovora*). They observed that the radio sensitivity to gamma rays was genotype dependent and the decrease in regeneration was not proportional to the dose.

2.4 Production of toxic metabolites from *Phytophthora* spp. and partial purification of the metabolites

Wolf (1953) reported that filtrate derived from the cultures of *P. parasitica* var. *nicotianae* grown in potato dextrose broth for four weeks or longer induced wilting on detached leaves of tobacco and tomato.

Wolf and Wolf (1954) determined the extent of toxin production of *P. parasitica* var. *nicotianae* on the basis of the amount of wilting induced on detached leaves of tobacco and tomato. Wilting occurred in dilutions of 1:1, 1:5 and 1:10. This toxin was moderately heat stable, non volatile and dialyzable.

Forest and Steib (1961) observed involvement of toxic metabolites in seed piece rot of sugarcane. Sugarcane seed pieces planted in vermiculite were watered with extracts from *Rhizactonia* spp. and other rotting organisms like *Phytophthora erythroseptica* Pethyb, *Phytophthora megasperma* and *Glomerella tucumanensis*. Seedlings watered with culture filtrate of *P. erythroseptica* for five days killed 100 per cent seedlings while less concentrated extracts caused different degrees of wilting.

Seidel (1961) studied the nutrient requirement and toxin formation of the fungus *P. infestans* in different synthetic nutrient solutions. He found that NH_4 and tartarate were the essential factors in the nutrient solutions for production of toxin and of five sugars tested, sucrose proved to be the best sugar for protein production. He also reported that boiling the filtrate led to a marked reduction in the toxicity of the culture filtrate.

Savel'eva and Rubin (1963) reported that *P. infestans* produced a polysaccharide in the liquid culture which is toxic to the plants. Savel'eva and Vasyukova (1966) observed that *P. infestans* produced two types of toxins. They were polysaccharides isolated from the protein lipid polysaccharide complex and the culture medium and proteins from the mycelium. Acetylized amino saccharides were detected in the polysaccharide from the medium which was more toxic than that from the mycelium.

Paxton (1972) reported toxin production by *Phytophthora megasperma* Drechst. var. *Sojae* Hild in soyabean broth. The toxin accumulated in an incubation period of seven days caused wilting of one week old seedlings of the cultivar Harosoy 63 soyabeans. Wilting occurred in plants without roots indicating that the toxin effect was not primarily on the root system. He further reported that the toxin was water soluble, stable after autoclaving and did not pass through dialysing membranes.

Ballio *et al.* (1972) observed toxin production of *Phytophthora nicotianae* B. de Haan var. *parasitica* on synthetic medium containing sucrose, L-asparagine mineral salts and vitamins. Phytotoxicity of culture filtrates reached its maximum when shake cultures had grown for 60 to 90 h at 27°C in stirred fermenters.

Lee (1973) reported that *P. palmivora* produced a toxin in liquid cultures and the symptoms produced by the inoculation of toxin were the same as that of the pathogen. This result was further confirmed by Vilasini (1982).

Phytotoxicity of water soluble ß 1-3 glucans from *Phytophthora cinnamomi, P. palmivora* and *P. megasperma* var. *Sojae* was reported by Keen *et al.* (1975). ß 1-3 glucans produced wilting symptoms on *Persea indica*, soyabean, cocoa and tomato at 0.01 to 0.5 mg/ml.

Behnke and Lonnendinker (1977) isolated phytotoxic substances from the culture filtrates of the fungus *P. infestans.*

Phytophthora cryptogea Pethybr and Laff grown on glucose-glutamate medium in shake cultures produced toxic metabolites and caused laminar necrosis, growth inhibition and death of tobacco plants (Csinos and Hendrix, 1977). The severity of growth inhibition and foliar necrosis increased with the duration of exposure of the roots to the extracts and long exposures (60 minutes) often resulted in plant death.

Csinos and Hendrix (1977) reported that *P. crytogea* produced exotoxin and endotoxin. Sterilized aqueous extract of the mycelium and culture filtrate of the pathogen when administered on excised tobacco leaves, caused water soaking with in 12 h, laminar collapse with in 20 h and extensive dehydration with in 48 h.

Csinos and Hendrix (1978) reported the effect of culture extracts from 53 isolates of *Phythium* and *Phytophthora* on excised tobacco leaves. They found that toxic extracts were obtained from most isolates of *P. crytogea, P. drechsleri, P. erythroseptica* and *P. megasperma* not reported to be parasitic on tobacco.

Cell free culture filtrates of *P. palmivora* MF_4 induced vascular browning and flaccidity of the cut shoots of black pepper indicating the involvement of toxins (CPCRI, 1979).

Plich and Rudnicki (1979) reported that filtrates from 14 day old culture of *P. cactorum* contained a toxin which induced wilting of tomato leaves after six to eight h and stem after 15 to 24 h. They also observed maximum toxin production in a medium containing asparagine as a nitrogen source at temperature ranging from 24 to 26°C and in the dark. The toxin was stable when autoclaved at 121°C in strong

acid but not in strong base. It was non-dialysable had a relatively high molecular weight and was hydrophobic.

Woodward *et al.* (1980) observed that wilt inducing toxins from *Phytophthora* spp contained ß glucans and glucan hydrolases.

2.4.1 Partial purification of toxic metabolites from *Phytophthora* spp.

Production of phytotoxins by *Phytophthora nicotianae* and partial purification of the toxic metabolites by column chromatography was reported by Ballio *et al.* (1972). The culture filtrates when treated with 7 per cent ethanol removed material that was inactive. Column chromatography in 'Sephadex G 25 Fine' was chosen for the first stages of purification. Further fractionation on DEAE sephadex gave two groups of active fractions which caused different symptoms on leaves of tomato cuttings. Similar results were obtained when CM sephadex was used for the second step in purification. It was understood that more than one phytotoxin was present in the culture filtrates of *P. parasitica*. Chemical nature of active compounds when examined revealed that one of them was a polysaccharide.

Savel'eva and Rubin (1963) reported that the polysaccharide secreted by the fungus *P. infestans* into the culture medium could be separated into three components. The polysaccharide and its components were toxic to leaves and tubers of potato.

The extraction of ^{14}C mycolaminaran from *Persea indica* seedlings by ion exchange chromatography was described by Keen *et al.* (1975).

A non pectolytic protein from *Phytophthora capsici* that macerates plant tissue was reported by Yoshikawa *et al.* (1977). The macerating factor was isolated

from culture filtrate of *Phytophthora capsici* by successive column chromotography on Sephadex G 100, hydroxylapatite cellulose and diethyl amino ethyl (DEAE) cellulose. The specific activity of the macerating factor based on protein content, increased 430 fold during the purification procedure. The macerating factor appeared to have a high molecular weight (close to or over 150,000) based on Sephadex G 100 gel filtration. The pH optimum for maceration of cucumber pericarp by the factor was 6.0 to 6.25 and its macerating activity was completely destroyed by heat (70°C for 10 minutes) or trypsin treatments. The purified factor had a maximum UV absorption at 278 nm and is a thermolabile protein possibly an enzyme with an unknown substrate.

Partial purification of toxin from culture filtrate of *Phytophthora citrophthora* and the assays for determining the effect of partially purified toxins (PPT) on protoplasts was reported by Breiman and Galun (1981). Out of the three methods tried viz. fluorescin diacetate (FDA) viability staining, colony formation and ^{14}C amino acid uptake, inhibition of ^{14}C amino acid uptake proved to be the most sensitive method for quantifying PPT.

The isolation and purification of mycolaminaran from the cytoplasm of *P. megasperma* and its inhibition effect on initial viral infection on certain *Nicotiana* species was reported by Zinnen *et al.* (1991). Further characterization of mycolaminaran induced resistance against TMV, CMV and Tomato spotted wilt virus was reported by Heinkel *et al.* (1992).

Materials and Methods

MATERIALS AND METHODS

The present investigations were carried out at the Plant Tissue Culture Laboratory of the Department of Plantation Crops and Spices, College of Horticulture, Kerala Agricultural University, Vellanikkara, Thrissur during September 1991 to January 1995. The study was aimed at exploiting somaclonal variation in black pepper (*Piper nigrum* L.) and developing screening procedures for *Phytophthora* foot rot resistance/tolerance using tissue culture techniques. The details regarding the experimental materials, methodology adopted for conducting the various aspects of the study are presented in this chapter. The procedure reported for callus mediated organogenesis in black pepper by Nazeem *et al.* (1990) was employed to produce regenerants from leaf calli.

3.1 Source of explants

The explant sources used for the study included rooted cuttings of *Piper nigrum* cultivars - Panniyur-1, Karimunda, Cheriakanyakkadan, Balankotta, Kalluvally, Panniyur-2 and *P. colubrinum* (a species of *Piper* reported to be immune to *Phytophthora* foot rot) and *in vitro* germinated seedlings of the cultivars Panniyur-1, Karimunda, Cheriakanyakkadan, Balankotta and Kalluvally. In addition to these, another culture found to be highly responding under *in vitro* conditions, isolated in the tissue culture laboratory of the Department of Plantation Crops and Spices (Culture 1) was also used to study some of the aspects of this investigation.

3.1.1 Rooted cuttings

Runners were collected from grown up vines of the cultivars maintained at the Pepper Research Station, Panniyur. The cuttings were rooted after giving a dip in IBA 1000 ppm for 45 seconds and maintained in pots in the glass house of the College of Horticulture. In order to reduce the contamination *in vitro*, prophylactic sprays were given to these plants with Bavistin and Ekalux 0.1 per cent at fortnightly intervals.

3.1.2 *In vitro* seedlings

Ripe berries were collected from grown up vines of the five cultivars maintained at the Pepper Research Station, Panniyur. After removing the pulp, the seeds were thoroughly washed in tap water and dried for one day in shade. The seeds were then surface sterilised with $HgCl_2$ (0.1 per cent) for 8 minutes in a laminar air flow cabinet and were washed free of $HgCl_2$ by rinsing with three changes of sterile distilled water. The seeds were dried on sterile blotting paper and were sown in sterile wet sand and vermiculite medium taken in equal proportions. The cultures were incubated at 26 $\pm$ 1 $^{\circ}$C in darkness till the seeds started germination (Plate 1).

3.2 Culture medium

3.2.1 Materials

The major and minor elements required for the preparation of media were of analytical grade and procured from M/s.BDH Laboratories, Sisco Research Laboratories (SRL) and Merck, India. The amino acids, vitamins and plant growth regulators were obtained from M/s.Merck and Sigma Chemicals, USA.

Plate 1. A seedling of black pepper raised *in vitro* in sand + vermiculite medium

3.2.2 Glasswares

Borosilicate glasswares of corning/borosil brand were used for the experiment.

3.2.3 Composition of the media

Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), Ribeiro's medium (Ribeiro, 1978), Potato Dextrose Agar medium and Carrot Agar medium were used in the present investigations. The composition of these media are given in Appendix I.

3.2.4 Preparation of the medium

Standard procedures (Gamborg and Shyluk, 1981) were followed for the preparation of the MS medium. The pH of the medium was adjusted between 5.6 to 5.8 using 0.1 N NaOH or 0.1 N HCl. The medium was solidified with 0.7 per cent good quality agar and distributed to small test tubes (15 x 2.5 cm) at the rate of 15 ml each, to bigger test tubes (200 mm x 38 mm) at the rate of 60 ml each and to jars (250 ml) at the rate of 50 ml each. The test tubes were plugged with non-absorbent cotton and the jars plugged with polypropylene caps, the centre of which was provided with plugs of non-absorbent cotton. Autoclaving was done at 121 °C at 15 psi (1.06 kg/cm^2) for 20 minutes (Dodds and Roberts, 1982). The medium was allowed to cool to room temperature and stored in the culture room until used.

Ribeiro's liquid medium was prepared as reported by Ribeiro (1978). After adjusting the pH of the medium to 6.2 with 6 N KOH the medium was

distributed to 100 ml Erlenmeyer flask at the rate of 50 ml per flask and autoclaved for 15 minutes at 15 psi.

3.3 Transfer area and aseptic manipulations

All the aseptic manipulations were carried out under the hood of a clean laminar air flow cabinet (Klenzaids).

3.4 Culture conditions

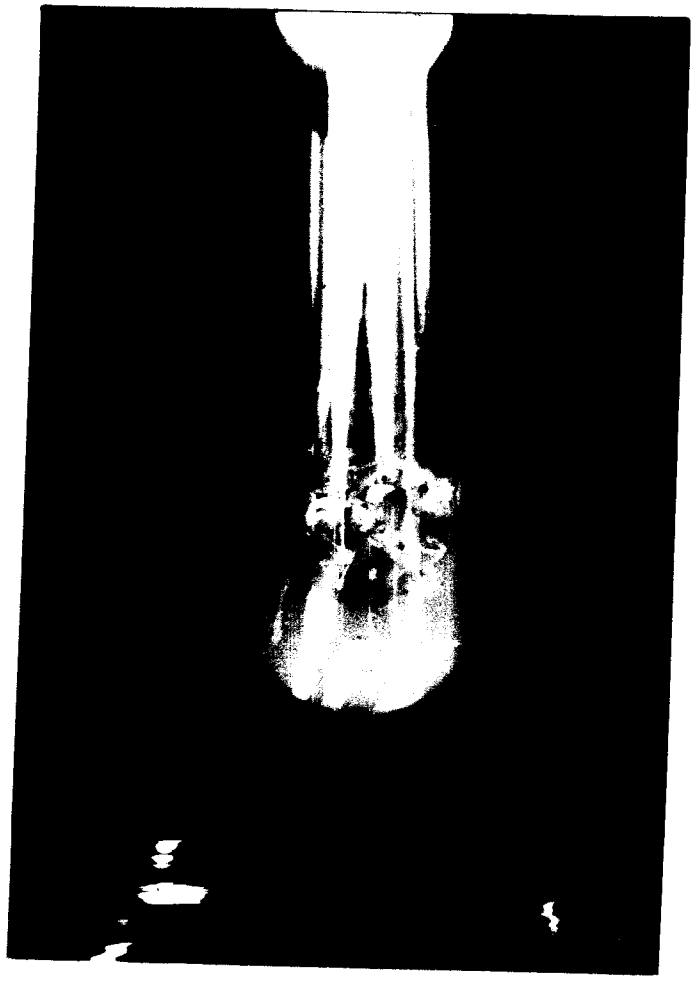
The cultures were incubated at 26 $\pm$ 1 $^{\circ}$C in an air conditioned culture room with 16 h photoperiod (1000 lux) supplied by fluorescent tubes unless otherwise mentioned. Humidity in the culture room varied between 60 to 80 per cent according to the climatic conditions prevailed.

3.5 Induction of calli

Calli were induced on stem and leaf segments of *in vitro* seedlings and basal leaf segments of mature leaves taken from rooted cuttings of the different cultivars and *P. colubrinum*.

Stem and leaf explants of *in vitro* seedlings were surface sterilised with 0.1 per cent $HgCl_2$ for three minutes and mature leaf segments for 8 minutes. After surface sterilisation, the explants were washed free off the sterilant, dried over sterile blotting paper and inoculated into MS medium supplemented with sucrose 3 per cent, IAA and BAP 1.0 mg l^{-1}. After three weeks of incubation, the induced calli were subcultured to the medium of the same composition for proliferation. The calli grown in the first subculture medium for two weeks were used for screening studies (Plate 2).

Plate 2. Cream nodular callus used for screening



3.6 Preparation and assay of culture filtrate

3.6.1 Influence of incubation period and type of culture on the accumulation of toxic metabolite(s) by *Phytophthora capsici*

The pure culture of *P. capsici* was collected from Indian Institute of Spices Research, Kozhikkode, Kerala. The stock isolate was maintained by periodic transfer on Carrot agar medium.

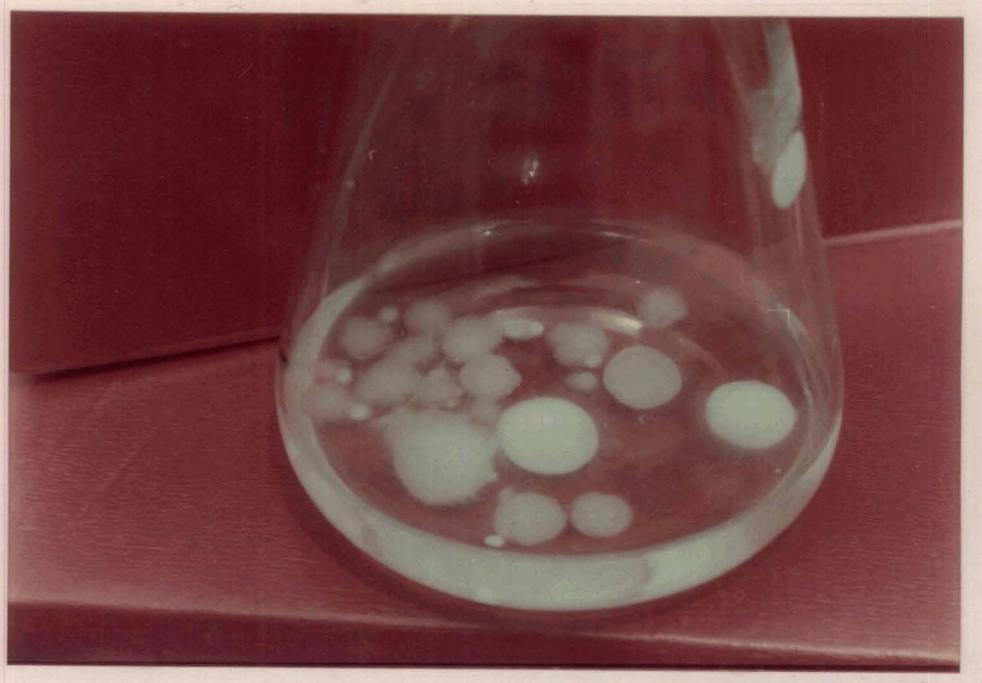
Liquid cultures of *P. capsici* were initiated in 50 ml of Ribeiro's medium by placing 10 mm culture discs of the seven day old cultures of the fungus grown in Potato dextrose agar medium (Plate 3). The cultures were given three different incubation periods (5 days, 10 days and 15 days) and two different conditions (shaking/stationary). Shaking was done in an orbital shaker cum incubator under continuous light maintained at a temperature of 25 °C. Stationary cultures were kept in the culture room.

The culture filtrate was collected by subsequent filtration through two layers of muslin cloth and Whatman No.1 filter paper. Half of the filtrate collected from each treatment was reduced to 1/10th of its volume by keeping in the laminar flow clean air chamber to get the concentrated culture filtrate (CCF). The accumulation of toxic metabolite(s) in culture filtrate of different treatments with and without reducing the volume was assessed using leaf puncture bioassay.

3.6.1.1 Leaf puncture bioassay of concentrated culture filtrate

Bioassay was done with the collected culture filtrate as such and with CCF of the different treatments, on detached leaves of the cultivar Karimunda, with four replications. The culture filtrate was dropped evenly on the lower surface of the

Plate 3. Liquid culture of *Phytophthora capsici* in Ribeiro's medium



leaves after gently pricking the area with sterile needle. The treated leaves were placed on flower pins immersed in water and covered with a bell jar containing moist cotton to provide high humidity and were observed for the development of necrotic spots. The diameter of lesions formed was measured in two directions perpendicular to each other 60 h after inoculation. The average lesion diameter was correlated with the accumulation of the toxic metabolite(s).

3.6.2 Effect of autoclaving of concentrated culture filtrate on symptom development

Concentrated culture filtrate collected from the shaken cultures of three incubation periods were autoclaved at 15 psi pressure for 20 minutes. The autoclaved CCF was bioassayed using detached leaves of the cultivar Karimunda and the average diameter of the lesions formed was noted.

3.6.3 Effect of dilution of concentrated culture filtrate on symptom development

Concentrated culture filtrate collected from the shaken cultures of ten day incubation period was diluted with sterile distilled water to 25, 50 and 75 per cent v/v. The diluted culture filtrate was tested for the development of symptoms on detached leaves of the cultivar Karimunda. The average diameter of the lesions formed were recorded and compared with the lesions formed by the inoculation of 100 per cent v/v CCF.

3.6.4 Host-specificity of the concentrated culture filtrate on symptom development

The toxin produced by *P. capsici* was reported to be non-specific (Sarma *et al.*, 1991). Inorder to test the specificity of the collected CCF, bioassay was done

on detached leaves of crops like tomato, cinnamon, clove, amaranth, chillies, brinjal, *P. colubrinum, P. nigrum* and *P. longum*. Observations on development of symptoms and nature of symptoms were noted.

3.6.5 Electrolyte leakage assay of concentrated culture filtrate

Electrolyte leakage studies were conducted on mature leaves, calli induced from mature leaf segments and calli from *in vitro* seedling explants of the five cultivars. Leaves and calluses for electrolyte leakage studies were prepared as described by Vidyasekharan *et al.* (1986) and (1990) respectively. Conductance of ambient solutions was measured in μmhos at intervals of 10 minutes with Model 120 Orion conductivity bridge using a dip type cell. The pattern of electrolyte leakage from leaves and calluses were observed.

3.6.6 Electrolyte leakage from calli at different dilutions of concentrated culture filtrate

Concentrated culture filtrate was diluted to 2.5, 5, 7.5 and 10 per cent v/v and the electrolyte leakage induced from calli of *in vitro* seedling explants was recorded in μmhos at intervals of 10 minutes.

3.7 *In vitro* screening of calli using concentrated culture filtrate

3.7.1 Standardisation of the level of CCF for *in vitro* callus screening

Concentrated culture filtrate was incorporated to modified MS medium (½ MS supplemented with IAA and BAP 1.0 mg l^{-1}) at varying levels (2.5, 5, 7.5 and 10% v/v). Callus pieces (5-10 mm) of the five cultivars from both the sources were inoculated after cutting and washing with sterile distilled water and incubated for a period of three weeks. The cultures were kept in dark for the first three days

and there after under light. Callus necrosis was taken as an index to measure the toxin sensitivity. The necrosis of the calli at different levels of CCF was assessed and the percentage of necrotic calli was calculated. Modified MS medium to which concentrated Ribeiro's medium was added to the same volume as that of CCF served as the control (medium control).

3.7.2 Effect of concentrated culture filtrate on callus growth and proliferation

Growth and proliferation of calli of different cultivars, from both the sources, at 7.5% v/v of CCF were assessed. The growth score was done based on the spread of the calli and maximum score of 3 was given for those that showed good growth occupying the whole surface of the media three weeks after incubation. The callus growth index (CGI) was calculated as

$$CGI = P_1 \times G$$

Where P_1 is the percentage of surviving calli and G is the growth score. The callus growth index in the CCF added medium was compared with the growth index in the medium control.

3.8 Effect of concentrated culture filtrate at various stages of development of calli

3.8.1 Effect of prolonged duration of selection with concentrated culture filtrate on shoot regeneration from calli

Concentrated culture filtrate was incorporated to the modified MS medium (½ MS supplemented with IAA and BAP 1.0 mg l^{-1}) at the rate of 7.5 per cent v/v. Three to four weeks old calli induced from both the sources were inoculated into the medium and observed for regeneration of shoots for three more

subcultures each with an incubation period of three weeks. The percentage of calli showing regeneration of shoots was observed and compared with the medium control.

3.8.2 Effect of concentrated culture filtrate on proliferation of shoots

Concentrated culture filtrate was incorporated to the modified MS medium (½ MS supplemented with IAA and BAP 1.0 mg l^{-1}) at the rate of 7.5 per cent v/v. Already regenerated cultures of the five different cultivars in normal media were transferred to CCF incorporated media. The effect of CCF on further proliferation of shoots was observed one month after inoculation and compared with the medium control.

3.8.3 Effect of concentrated culture filtrate on shoot growth

Concentrated culture filtrate was incorporated at 7.5 per cent v/v to the elongation medium (½ MS supplemented with IAA 0.1 mg l^{-1} and BAP 0.2 mg l^{-1}) and uniform shoots of the cultivars viz., Kalluvally, Balankotta, Cheriakanykkadan and Karimumda were inoculated and incubated for a period of one month. Elongation of the shoots and the rate of leaf and node production of the different cultivars were observed one month after inoculation and compared to that in the medium control.

3.8.4 Effect of concentrated culture filtrate on rooting and root growth

Concentrated culture filtrate was incorporated 7.5 per cent v/v to the rooting medium (½ MS supplemented with 2% sucrose and IBA 1.0 mg l^{-1}) and uniform shoots of the cultivars viz., Kalluvally and Cheriakanyakkadan were

inoculated to the rooting medium after giving a pulse treatment for one minute in IBA 1000 ppm prepared in absolute alcohol. The number of roots produced, the length of roots and thickness of the roots (diameter) were observed 40 days after inoculation and compared with the medium control.

3.9 Direct selection of the calli by the different screening methods

Direct screening of calli was adopted by the following three methods. Calli after initial screening, were transferred to normal regeneration medium free of CCF. Only calli induced from *in vitro* seedling explants were subjected to study.

3.9.1 Method 1 (Screening the calli by growing in concentrated culture filtrate incorporated modified MS medium)

The concentrated culture filtrate was incorporated to the modified MS medium @ 7.5% v/v. Callus pieces (5-10 mm) induced from *in vitro* seedling explants of the five cultivars and the highly responding culture (culture No.1) were inoculated to the medium and incubated for a period of three weeks. The callus bits grown on modified MS medium to which concentrated Ribeiro's medium was added @ 7.5 per cent v/v served as control. The surviving calli from both the treatments were sub cultured to normal regeneration medium (½ MS supplemented with IAA and BAP 1.0 mg l^{-1}) free of CCF.

3.9.1.1 Effect of callus screening (Method 1) on regeneration of shoots

The calli after screening by method 1 were transferred to modified MS medium free of CCF and supplemented with IAA and BAP 1.0 mg l^{-1}. The percentage of cultures showing regeneration of shoots was observed and compared with the regeneration from unscreened calli.

3.9.1.2 Effect of callus screening (Method 1) on proliferation of shoots

The regenerated cultures from screened and unscreened calli were transferred to modified MS medium free of CCF and supplemented with IAA and BAP 1.0 mg l^{-1} for further proliferation of shoots. The number of shoots proliferated in the 2nd subculture from the screened and unscreened calli were observed.

3.9.1.3 Effect of callus screening (Method 1) on the recovery of rootable shoots

The regenerants from screened and unscreened calli were transferred to modified MS medium supplemented with IAA 0.1 mg l^{-1} and BAP 0.2 mg l^{-1} for elongation. The recovery of rootable shoots in the 5th subculture was compared.

3.9.1.4 Effect of callus screening (Method 1) on root growth

The rootable shoots from the screened and unscreened cultures of the different cultivars were rooted in modified MS medium supplemented with sucrose 2.0 per cent and IBA 1.0 mg l^{-1}. Days taken for induction of root initials, the number of roots produced, length of roots and thickness of roots of the plantlets from the two sources were compared.

3.9.1.5 Comparison of the anatomy of the roots regenerated from screened and unscreened calli

The anatomy of the roots regenerated from the screened (Method 1) and unscreened calli of cultivars like Kalluvally, Balankotta, Cheriakanyakkadan and Karimunda were compared. Transverse sections of the roots were taken, stained with safranine and the anatomy of the roots were observed under a binocular

research microscope. Photomicrographs were taken using Getner photomicrographic unit.

3.9.2 Method 2 (Screening the calli by shaking in concentrated culture filtrate)

3.9.2.1 Standardisation of the level of CCF and duration of shaking for screening by method 2

Calli induced from *in vitro* seedling explants (less than 5 mm size) of the five cultivars were transferred to modified liquid MS medium containing CCF at varying levels (10 and 15% v/v) and incubated in a shaker cum incubator for two different incubation periods viz. 48 and 72 h. The calli were then transferred to the modified MS solid medium free of CCF. The percentage of necrotic calli at different levels of CCF for the two incubation periods for the five cultivars were noted. Callus pieces shaken in modified MS liquid medium to which concentrated Ribeiro's medium was added at the same volume as that of CCF served as control.

3.9.2.2 Effect of callus screening (Method 2) on various stages of development of cultures

Effect of initial screening the calli by (Method 2) on various stages of development like regeneration of shoots, proliferation of shoots, recovery of root-able shoots and rooting and root growth were compared with that of the cultures from unscreened calli.

3.9.3 Method 3 (Screening the calli by double layer culture technique)

3.9.3.1 Effect of callus screening (Method 3) on callus necrosis

The double layer culture technique utilized in microbiology (Lepoivre *et al.*, 1986) was modified. Jars containing the Ribeiro's medium with toxic

metabolite(s) was over layered with modified MS medium each at a thickness of 20 mm and were incubated for a period of seven days for the diffusion of the toxic materials. Three weeks old cream nodular calli (5-10 mm) were inoculated on the upper medium at the rate of 10 calli/jar. The toxin sensitivity of the different cultivars was assessed on the basis of necrosis of the calli, three weeks after inoculation. Callus pieces inoculated on double layer without the toxic metabolites served as control. The surviving calli of both the treatments were transferred to normal regeneration medium.

3.9.3.2 Effect of callus screening (Method 3) on various stages of development of cultures

Effect of screening the calli by double layer culture on various stages of development like regeneration of shoots, proliferation of shoots, recovery of rootable shoots, rooting and root growth was observed and compared with that of the cultures from unscreened calli.

3.10 *In vitro* induction of mutation using gamma irradiation

A ^{60}Co source (Gamma chamber 900 of BARC, Mumbai) with a dose rate of 830 Gy/h was used for irradiation.

3.10.1 Effect of gamma irradiation on callus growth (Panniyur-2)

Calli induced from mature leaf segments of the variety Panniyur-2 were made into uniform pieces, washed thoroughly in sterile distilled water, dried over sterile blotting paper and subjected to gamma irradiation of doses 2.5, 5.0, 7.5, 10.0, 15.0, 20.0, 50.0 and 100.0 Gy. After irradiation, the callus pieces were

immediately transferred to fresh modified MS medium and the percentage of cultures putting forth growth and the growth of the calli at different doses were evaluated.

3.10.2 Effect of gamma irradiation on callus growth (cv. Kalluvally)

The calli induced from mature leaf segments of the cultivar Kalluvally were subjected to gamma irradiation of doses 15.0, 17.5, 20.0, 22.5 and 25.0 Gy. The percentage of calli putting forth growth and the growth of the calli in the modified MS medium were observed.

3.10.3 Standardisation of the gamma irradiation dose for callus growth and regeneration in black pepper cultivars

The calli induced from mature leaf segments of the cultivars Kalluvally, Karimunda, Panniyur-1 and Panniyur-2 were subjected to gamma irradiation doses 10.0, 20.0, 30.0, 40.0 and 50.0 Gy. The growth of the calli and regeneration of shoots from the calli in modified MS medium were noted.

3.10.4 Effect of gamma irradiation (30 Gy) on callus growth and regeneration of shoots (calli from *in vitro* seedling explants)

Calli induced from *in vitro* seedling explants of the cultivars Cheria-kanyakkadan, Karimunda, Kalluvally and Balankotta were subjected to gamma irradiation of 30 Gy. The percentage of calli putting forth growth, nature of calli and shoot regeneration from calli were observed.

3.10.5 *In vitro* screening of the irradiated calli using concentrated culture filtrate

The irradiated calli from *in vitro* seedling explants were subjected to

screening by growing in 7.5 and 10 per cent v/v CCF incorporated modified MS medium. The percentage of cultures putting forth callus growth, percentage cultures showing shoot regeneration and nature of shoots produced were compared with the control.

3.11 Partial purification of the culture filtrate of *Phytophthora capsici*

In order to separate the toxic metabolite(s) from culture filtrates of *P. capsici* partial purification of the culture filtrate was attempted.

3.11.1 Separation of the toxic metabolite(s) from concentrated culture filtrate by organic solvent fractionation

Six organic solvents viz. methanol, acetone, dichloro ethane, diethyl ether, chloroform and ethyl acetate were tried to separate the toxic metabolite(s) from the concentrated culture filtrate. The solvent and aqueous layers were separated using a separating funnel. Both solvent and aqueous fractions were bioassayed using leaf puncture bioassay in the cultivar Karimunda.

3.11.2 Separation of the toxic metabolite(s) from concentrated culture filtrate by ion exchange chromatography

Separation of the toxic metabolite(s) from the concentrated culture filtrate of *P. capsici* was carried out using ion exchangers viz. Dowex 1 and Dowex 50. Strongly basic anion exchange resin Dowex 1 having dry mesh size 20 to 50 and strongly acidic cation exchange resin Dowex 50 (also known as HCR-W_2) with a dry mesh size 100-200 were used for the study. Five gram each of anion and cation exchange resins were suspended in distilled water overnight. On the following day Dowex 1 was repeatedly washed with 1N sodium acetate to make it in the basic form

The aqueous fraction left after separating the solvent fraction was concentrated and bioassayed using detached leaves of the cultivar Karimunda to verify the presence of toxic metabolite(s) if any in the aqueous fraction.

The coumarin standard was dissolved in small quantity of ethyl alcohol and the volume made up with sterile distilled water. This solution was also bioassayed in detached leaves of the cultivar Karimunda.

3.12 Production of somaclones without *in vitro* callus screening and screening the regenerants at whole plant level

3.12.1 Induction of calli

Calli were induced as described in 3.5.

3.12.1.1 Response of black pepper cultivars to callus induction and proliferation

Calli were induced on basal leaf segments of mature plants, stem and leaf explants of *in vitro* seedlings and leaf segments of *in vitro* seedling established cultures of different cultivars. The medium used for callus induction was MS medium at half the concentration supplemented with IAA and BAP 1.0 mg l^{-1}. Comparative performance of different cultivars and different explants on the time taken for callus induction, percentage of cultures showing callusing and proliferation of calli were observed. The callus index (CI) was worked out as

$$CI = P \times G$$

where P is the percentage of callus initiation and G is the growth score. A maximum score of three was given for those calli putting forth good growth covering the entire surface of the media.

3.12.1.2 Response of black pepper cultivars to shoot regeneration

The cream nodular calli induced in the modified MS medium (½ MS supplemented with IAA and BAP 1.0 mg l^{-1}) were subcultured to the same medium for further proliferation and regeneration of shoots. The response of different cultivars and the three source of explants were compared with respect to shoot regeneration in the 2nd subculture.

3.12.1.3 Effect of containers on shoot proliferation

Different containers viz., small culture tubes (15.0 x 2.5 cm), 100 ml Erlenmeyer flask, 250 ml Erlen- meyer flask, big culture tubes (380 x 200 mm) and 250 ml jars were compared for the proliferation of shoots by inoculating uniform pieces of the already regenerated cultures of the cultivar Karimunda and noting the further proliferation of shoots.

3.12.1.4 Response of black pepper cultivars to the proliferation of shoots

The induced shoots of the different cultivars were subcultured to the same modified MS medium for further proliferation of shoots. The response of different cultivars was compared with respect to proliferation of shoots in the 3rd subculture.

3.12.1.5 Response of black pepper cultivars to the recovery of rootable shoots

The cultivars with the proliferating shoots were transferred to the elongation medium (½ MS supplemented with IAA 0.1 mg l^{-1} and BAP 0.2 mg l^{-1}) for

the 4th, 5th, and 6th subcultures. The response of different cultivars in the recovery of rootable shoots was compared.

3.12.1.6 Response of black pepper cultivars to rooting and root growth

The rootable shoots were transferred to rooting medium (½ MS supplemented with 2% sucrose and IBA 1 mg l^{-1}) after giving a pulse treatment with IBA 1000 mg l^{-1} prepared in absolute alcohol. The response of different cultivars was compared with respect to percentage of rooting, days taken for root induction, length, number and thickness of roots.

3.13 Planting out, hardening and final survival

3.13.1 Effect of season and weather parameters on final survival of plantlets

The rooted plantlets produced in each month were hardened by exposing them in shade for two weeks in small pots filled with sterile sand and then transferred to polybags containing potting mixture made in the proportion of 1:1:1. The effect of season on the mortality of plants during and after hardening and on the final survival was assessed. Correlation with weather parameters such as maximum temperature, minimum temperature, number of rainy days, rainfall, mean relative humidity and sunshine hours on the mortality and final survival of plantlets were worked out.

3.13.2 Effect of callus screening on final survival of plantlets

The rooted plantlets produced from screened cultures of the different cultivars were observed for the mortality of plants during and after hardening and for the final survival and compared with that from unscreened cultures.

3.13.3 Influence of cultivars on final survival of plantlets

The rooted plantlets of different cultivars produced were compared with respect to the mortality of plants during and after hardening and final survival.

3.14 Screening the regenerated calliclones for tolerance/resistance to *Phytophthora capsici*

The calliclones produced after *in vitro* screening by the different methods and the clones derived from unscreened calli were further screened for tolerance/resistance to *P. capsici* by the following three methods.

3.14.1 Natural screening for resistance to *P. capsici* (by keeping in infected field)

Natural screening of regenerated calliclones for resistance to *P. capsici* was done during July-August 1994.

Severely infected field with *P. capsici* of the Pepper Research Scheme, Vellanikkara was utilized for this study. One row of healthy plants was kept in the middle of two rows of infected plants. Time taken for symptom development, the type of symptom development etc. were observed.

3.14.1.1 Effect of callus screening on the development of symptoms

Plants regenerated from screened and unscreened calli were compared for the development of symptoms by observing the number of plants taking infection on each day.

3.14.1.2 Effect of age of the calliclones on symptom development

Plants of the three age groups viz. three to six months, two to three months and one to two months were used for the study. Time taken for the symptom to develop and number of plants wilted on each day were compared between the three age groups.

3.14.1.3 Screening the regenerated calliclones by inducing electrolyte leakage with concentrated culture filtrate

Inorder to assess the tolerance/resistance level of the calliclones to the disease and to find out the influence of callus screening on electrolyte leakage, screening by electrolyte leakage method was attempted. Leaves of the clones produced from the screened and unscreened calli were collected at random and prepared for electrolyte leakage studies as described by Vidyasekharan *et al.* (1986).

3.14.1.3.1 Effect of callus screening on electrolyte leakage from regenerated calliclones

Ten calliclones of four cultivars viz. Kalluvally, Cheriakanyakkadan, Balankotta and Karimunda from screened and unscreened group were subjected to study. The electrolyte leakage from leaves of the regenerants produced from screened calli were compared with the leakage from leaves of the regenerants of the unscreened calli.

3.14.1.4 Screening the regenerated calliclones by artificial inoculation of culture disc of *P. capsici*

Screening the calliclones by the artificial inoculation of culture disc of *P. capsici* was also attempted to know the tolerance/resistance level of the clones

to the disease. The screening and scoring technique as reported by Kueh and Khew (1980b) for Phytophthora foot rot in black pepper was used with slight modification.

From the two month old regenerants established in the glass house, leaves of the same maturity were collected and using 5 mm culture disc of seven day old cultures of *P. capsici* inoculation was done to the lower surface of leaves after giving gentle pin prick with sterile needle.

The average diameter of the lesions developed 48 h after inoculation were observed and based on the average diameter of the lesion formed, the clones from screened and unscreened calli of different cultivars were grouped into five classes as shown below:

Class	Lesion diameter in cm
1	< 0.5 cm
2	0.5-1.0 cm
3	1.1-1.5 cm
4	1.6-2.0 cm
5	> 2.0 cm

Each class was given a score based on a scale ranging from 1 to 5 where 1 represented average lesion diameter < 0.5 cm, 2 = 0.5-1.0 cm, 3 = 1.1-1.5 cm, 4 = 1.6-2.0 cm and 5 = > 2.0 cm. Lesion diameter score (LDS) was calculated by multiplying the percentage of plants coming under each class with the score given for the class.

Based on LDS, the effect of callus screening on the intensity of lesion development, the variation in intensity of lesion development among calliclones

within a cultivar and the effect of different methods of screening on the intensity of lesion development in the cultivar Kalluvally were evaluated.

3.15 Comparison of somaclonal variation in black pepper cultivars

Somaclonal variation was assessed in callus regenerants from a single seedling of each cultivar. The callus cultures were not given *in vitro* selection pressure with the toxic metabolite(s) of *P. capsici* and the cultures had undergone uniform number of subculture cycles to produce the plantlets.

3.15.1 Somaclonal variation in electrolyte leakage

Electrolyte leakage from leaves of ten callus regenerants of each of the cultivars (S_1-S_{10}) Kalluvally, Balankotta, Cheriakanyakkadan and Karimunda were studied to assess the somaclonal variation. Leaves for electrolyte leakage studies were prepared as described in 3.6.5.

3.15.2 Somaclonal variation in lesion development

Calliclones of two cultivars namely Kalluvally and Cheriakanyakkadan were assessed for the somaclonal variation in lesion development. The screening and scoring technique as reported by Kueh and Khew (1980) for *Phytophthora* foot rot in black pepper was used with slight modification as described in 3.14.1.4. Mean deviation from median was worked out as the measure of dispersion.

3.15.3 Effect of duration in culture on somaclonal variation in lesion development

The clones derived from a single callus culture (DL 99) were observed for the intensity of lesion development to know the effect of duration in culture on

somaclonal variation. The plantlets derived from cultures which had undergone seven to ten subculture cycles were observed for the intensity of lesion development and the measure of dispersion worked out.

3.16 Statistical analysis

Statistical analysis of the data recorded was carried out as per the techniques described by Panse and Sukhatme (1985).

Results

RESULTS

The results generated from the investigations conducted at the Plant Tissue Culture Laboratory of the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara are presented in this chapter.

4.1 Preparation and assay of culture filtrate

4.1.1 Influence of incubation period and type of culture on the accumulation of toxic metabolite(s) by *Phytophthora capsici*

The effect of period of incubation and type of culture on the accumulation of toxic metabolite(s) in culture filtrates of *P. capsici* were assessed based on the capacity of the culture filtrate to form lesions on detached leaves of the cultivar Karimunda. The average diameter of lesions formed 60 h after inoculation of culture filtrate is presented in Table 1.

The collected culture filtrate when bioassayed without reducing the volume did not produce symptoms in any of the treatments. But when the volume of the culture filtrate was reduced to 1/10th and bioassayed, symptoms were observed in shake cultures, for the different incubation periods (Plate 4). Among the two types of cultures tried, toxic metabolites were not accumulated in stationary cultures. In shake cultures, the highest accumulation of toxic metabolite(s) was noticed in T_9 (15 days incubation in shake cultures and culture filtrate reduced to 1/10th volume) which recorded a lesion diameter of 1.60 cm 60 h after inoculation. However, the treatments T_5 (10 day incubation in shake cultures and culture filtrate reduced to 1/10th volume) and T_9 were found to be on par.

Table 1. Influence of incubation period and type of culture on the accumulation of toxic metabolite(s) by *Phytophthora capsici*

	Treatments	Concentrated or not	Development of symptoms	*Diameter of lesion 60 h after inoculation (cm)
T_1	5 day incubation shaking	Concentrated	+	0.563
T_2	5 day incubation shaking	Not concentrated	-	-
T_3	5 day incubation stationary	Concentrated	-	-
T_4	5 day incubation stationary	Not concentrated	-	-
T_5	10 day incubation shaking	Concentrated	+	1.575
T_6	10 day incubation shaking	Not concentrated	-	-
T_7	10 day incubation stationary	Concentrated	-	-
T_8	10 day incubation stationary	Not concentrated	-	-
T_9	15 day incubation shaking	Concentrated	+	1.600
T_{10}	15 day incubation shaking	Not concentrated	-	-
T_{11}	15 day incubation stationary	Concentrated	-	-
T_{12}	15 day incubation stationary	Not concentrated	-	-
CD (0.05)				0.088
SEm±				0.027

* Average of four replications
+ indicates development of symptoms
(Culture filtrate was reduced to 1/10th of its volume. Leaf puncture bioassay was done in the cultivar Karimunda)

Plate 4. Comparison of symptoms induced by CCF, culture disc of *Phytophthora capsici* and medium control


CD
CCF

4.1.2 Effect of autoclaving of concentrated culture filtrate on symptom development

The results on the effect of autoclaving of CCF on development of symptoms on detached leaves of the cultivar Karimunda are presented in Table 2. The autoclaved CCF was found to produce typical symptoms as that of CCF without autoclaving in all the three incubation periods suggesting the thermostable nature of the toxic metabolite(s) (Plate 5). Even though the autoclaved CCF exhibited a slightly better symptom compared to CCF without autoclaving, both the treatments were on par at all the three incubation periods. Since the toxic metabolite(s) produced was found thermostable the concentration of the collected culture filtrate was done on hot plates maintained at 100°C to make CCF in further studies.

4.1.3 Effect of dilution of concentrated culture filtrate on symptom development

Highly significant variation was observed in lesion diameter when inoculated with CCF at different dilutions (Table 3). The highest lesion diameter of 1.59 cm was exhibited by 100 per cent CCF followed by 75 per cent CCF v/v (1.03 cm), 50 per cent CCF v/v (0.80 cm) and 25 per cent CCF v/v (0.51 cm). So the activity of toxic metabolite(s) was found to decrease at higher dilutions of CCF.

4.1.4 Host-specificity of concentrated culture filtrate on symptom development

The symptoms developed and the nature of symptoms produced by CCF on detached leaves of different crops are presented in Table 4. Symptoms were observed as brown spots on leaves of black pepper, long pepper, *P. colubrinum*, chillies, clove and brinjal, light brown spots in cinnamon and amaranth and

Table 2. Effect of autoclaving (15 psi for 20 minutes) of concentrated culture filtrate on development of symptoms

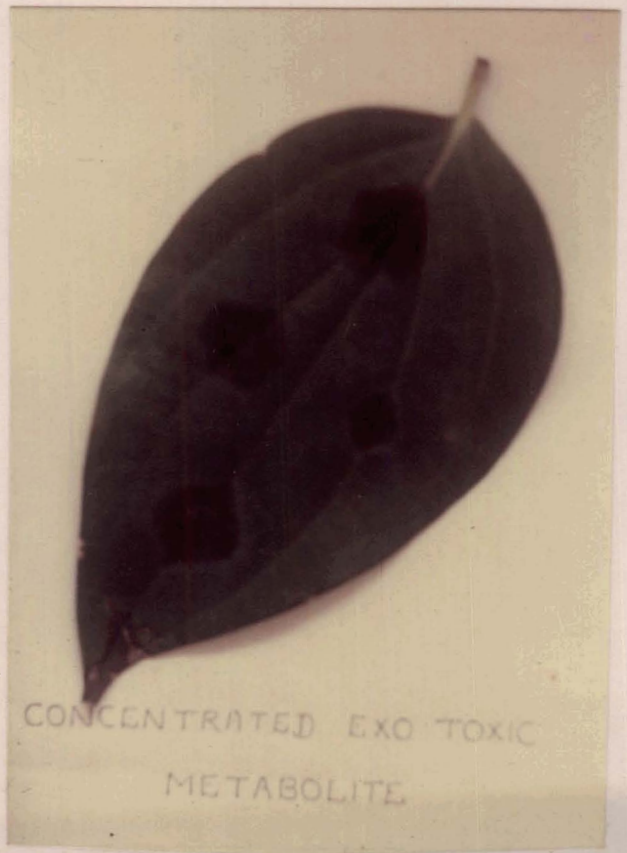
Treatments		Autoclaved or not	*Diameter of lesion in cm 60 h after inoculation
T_1	5 day incubation shaking culture	Autoclaved	0.600
T_2	5 day incubation shaking culture	Not autoclaved	0.563
T_3	10 day incubation shaking culture	Autoclaved	1.625
T_4	10 day incubation shaking culture	Not autoclaved	1.575
T_5	15 day incubation shaking culture	Autoclaved	1.637
T_6	15 day incubation shaking culture	Not autoclaved	1.600
CD (0.05)			0.0813
SEm±			0.027

* Average of 4 replications

Plate 5a. Symptom induced by autoclaved CCF

Plate 5b. Symptom induced by non-autoclaved CCF

CONCENTRATED HEAT STERILISED
EXO TOXIC METABOLITE



CONCENTRATED EXO TOXIC
METABOLITE

Table 3. Effect of dilution of concentrated culture filtrate on symptom development

Treatments	*Diameter of lesions in cm 60 h after inoculation
T_1 CCF at 25% v/v	0.513
T_2 CCF at 50% v/v	0.800
T_3 CCF at 75% v/v	1.025
T_4 CCF 100% v/v	1.587
CD (0.05)	0.0689
SEm±	0.022

*Average of 4 replications

Table 4. Host-specificity of concentrated culture filtrate on symptom development

Crops	Development of symptoms	Nature of symptoms
Tomato	+	Brownish black spots
Brinjal	+	Brown spots
Amaranth	+	Light brown spots
Chillies	+	Brown spots
Cinnamon	+	Light brown spots
Clove	+	Brown spots
Black pepper	+	Brown spots
Long pepper	+	Brown spots
Piper colubrinum	+	Brown spots

+ indicates the development of symptoms

brownish black spots in tomato. Since CCF produced symptoms in all the crops in which it was inoculated the toxic metabolite(s) present in CCF was found to be nonhost-specific in nature.

4.1.5 Electrolyte leakage assay of concentrated culture filtrate

When calluses and leaves were infiltrated with CCF of *P. capsici*, electrolyte leakage was induced both from leaves and calluses ten minutes after the treatment which increased with increase in time intervals (Table 5a and 5b; Fig.1 and 2). The electrolyte leakage from leaves and calluses followed the same pattern. The highest leakage was observed 20 minutes after the treatment, there after the difference in leakage showed a decreasing trend. So the leakage of electrolytes 20 minutes after the treatment was taken to evaluate the response of different cultivars.

Electrolyte leakage from leaves of different *P. nigrum* cultivars and *Piper colubrinum* showed highly significant variation (Table 6a and Fig. 3). The highest leakage of electrolytes over control was shown by Panniyur-1 (321.67 μmhos) followed by Karimunda (196.33 μmhos), Balankotta (186.67 μmhos), Cheriakanyakkadan (178.67 μmhos) and Kalluvally (169.67 μmhos). *Piper colubrinum* exhibited the lowest leakage of electrolytes (157.33 μmhos) about 104 per cent less than that from Panniyur-1. So the cultivars showing the same level of tolerance to *P. capsici* were exhibiting the same level of tolerance to CCF also.

Concentrated culture filtrate induced quick electrolyte leakage from calli similar to that from leaves showing the activity of CCF at callus/cellular level. The leakage exhibited by calli of different *P. nigrum* cultivars and *P. colubrinum* differed significantly (Table 6b and Fig.3). The lowest leakage was shown by calli

Table 5a. Electrolyte leakage induced by concentrated culture filtrate of *Phytophthora capsici* from leaves of black pepper cultivars

	Electrolyte leakage over control (μmhos)					
	Time (minutes)					
Cultivars	0	10	20	30	40	50
Panniyur-1	35	56	321	343	346	346
Karimunda	82	124	197	217	254	286
Kalluvally	83	113	169	175	183	206
Cheriakanyakkadan	48	84	178	217	222	222
Balankotta	90	130	186	215	250	272

Table 5b. Electrolyte leakage induced by concentrated culture filtrate of *Phytophthora capsici* from calli of black pepper cultivars

	Electrolyte leakage over control (μmhos)						
	Time (minutes)						
Cultivars	0	10	20	30	40	50	60
Panniyur-1	60	94	179	193	200	219	224
Karimunda	52	75	127	146	158	166	172
Kalluvally	31	51	81	92	130	140	140
Cheriakanyakkadan	17	48	83	103	108	118	122
Balankotta	41	63	102	141	168	189	201

Fig. 1 Electrolyte leakage from leaves of black pepper cultivars

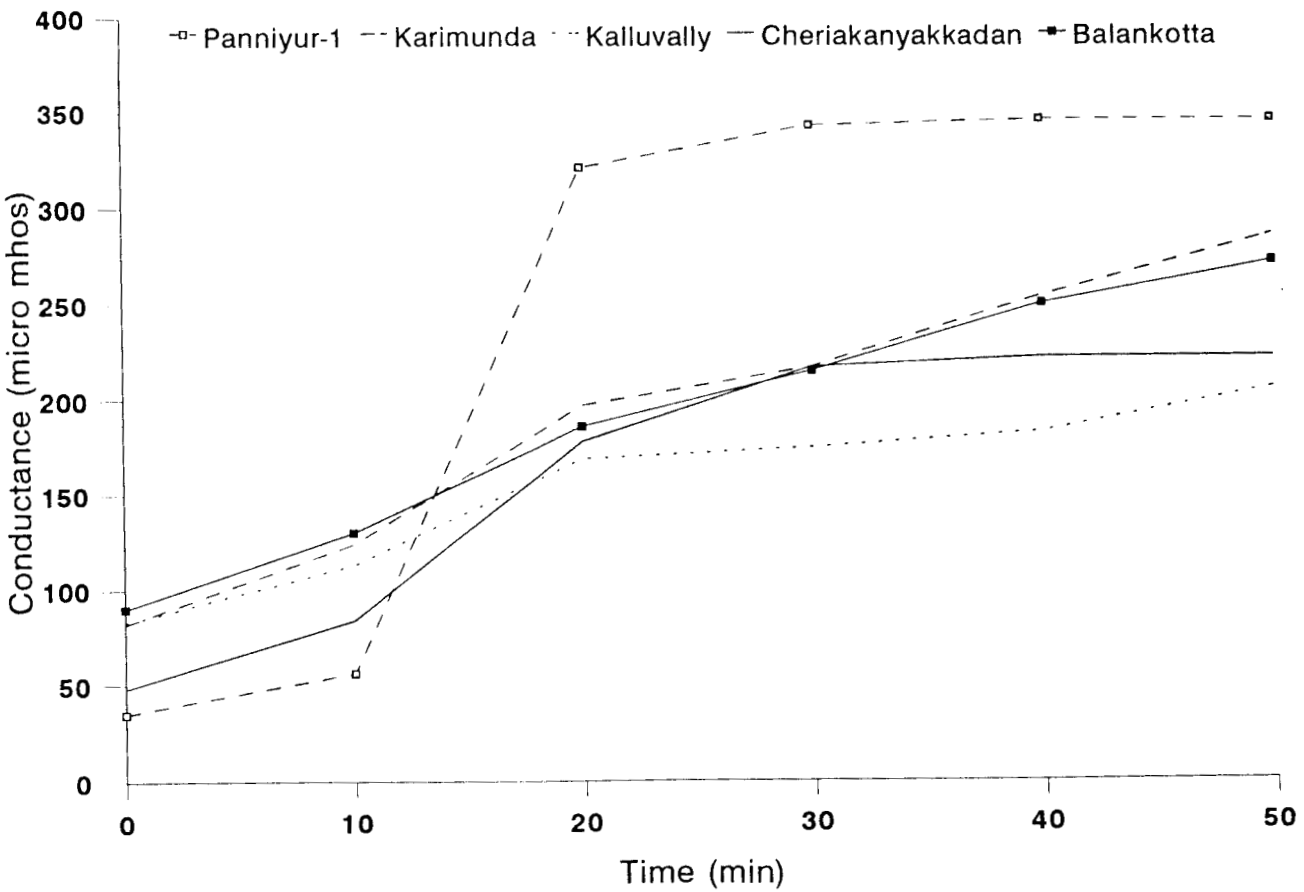


Fig. 2 Electrolyte leakage from calli of black pepper cultivars

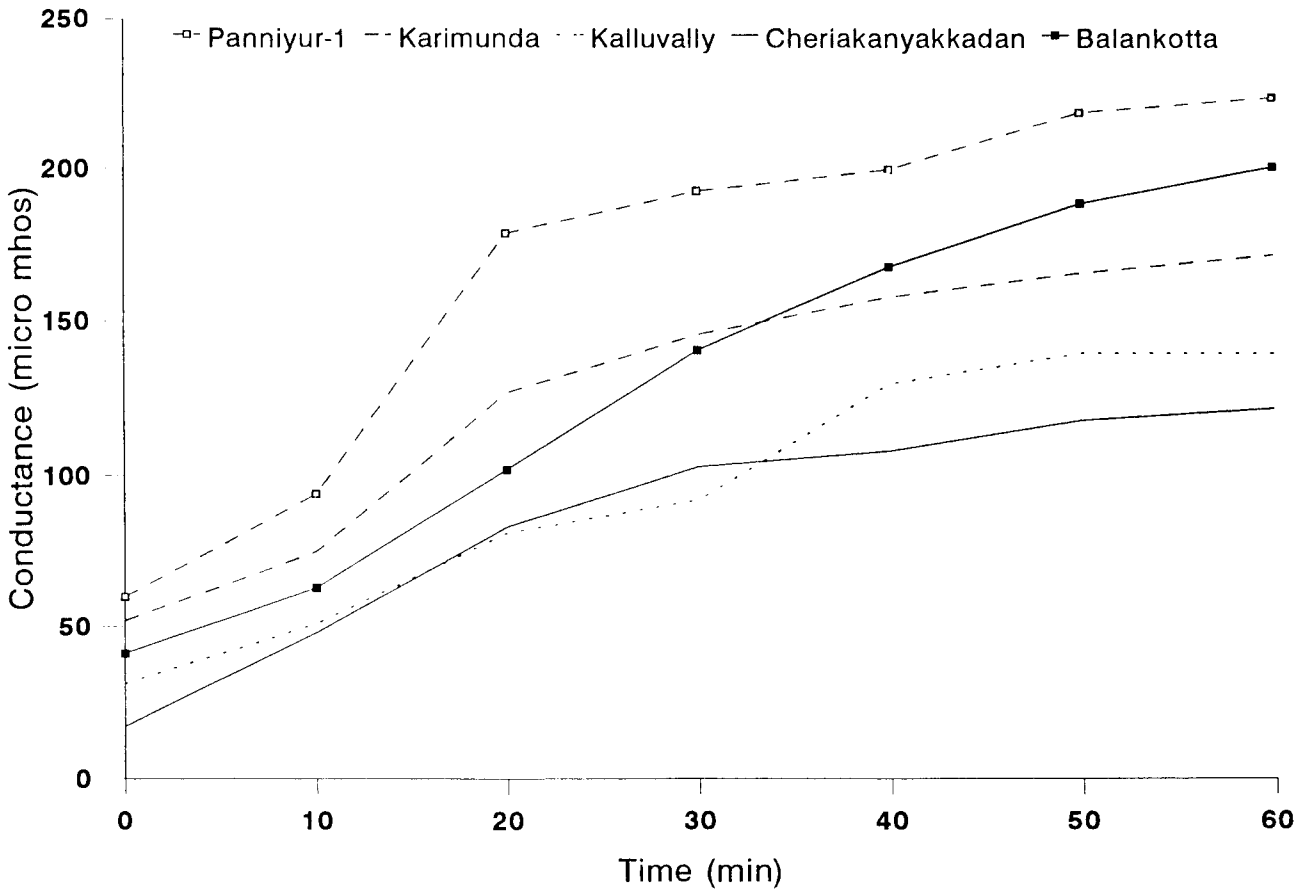


Table 6a. Electrolyte leakage induced by concentrated culture filtrate of *Phytophthora capsici* from leaves of *Piper nigrum* cultivars and *Piper colubrinum*

Genotype	*Leakage of electrolytes 20 minutes after the treatment (μmhos)	*Leakage over control (μmhos)
1. *P. colubrinum*	194.33	157.33
2. *Piper nigrum*		
a. Karimunda	242.33	196.33
b. Panniyur-I	355.66	321.67
c. Cheriakanikkadan	209.66	178.67
d. Kalluvally	200.66	169.67
e. Balankotta	216.67	186.67
CD (0.05)		4.172
SEm $\pm$		-1.35

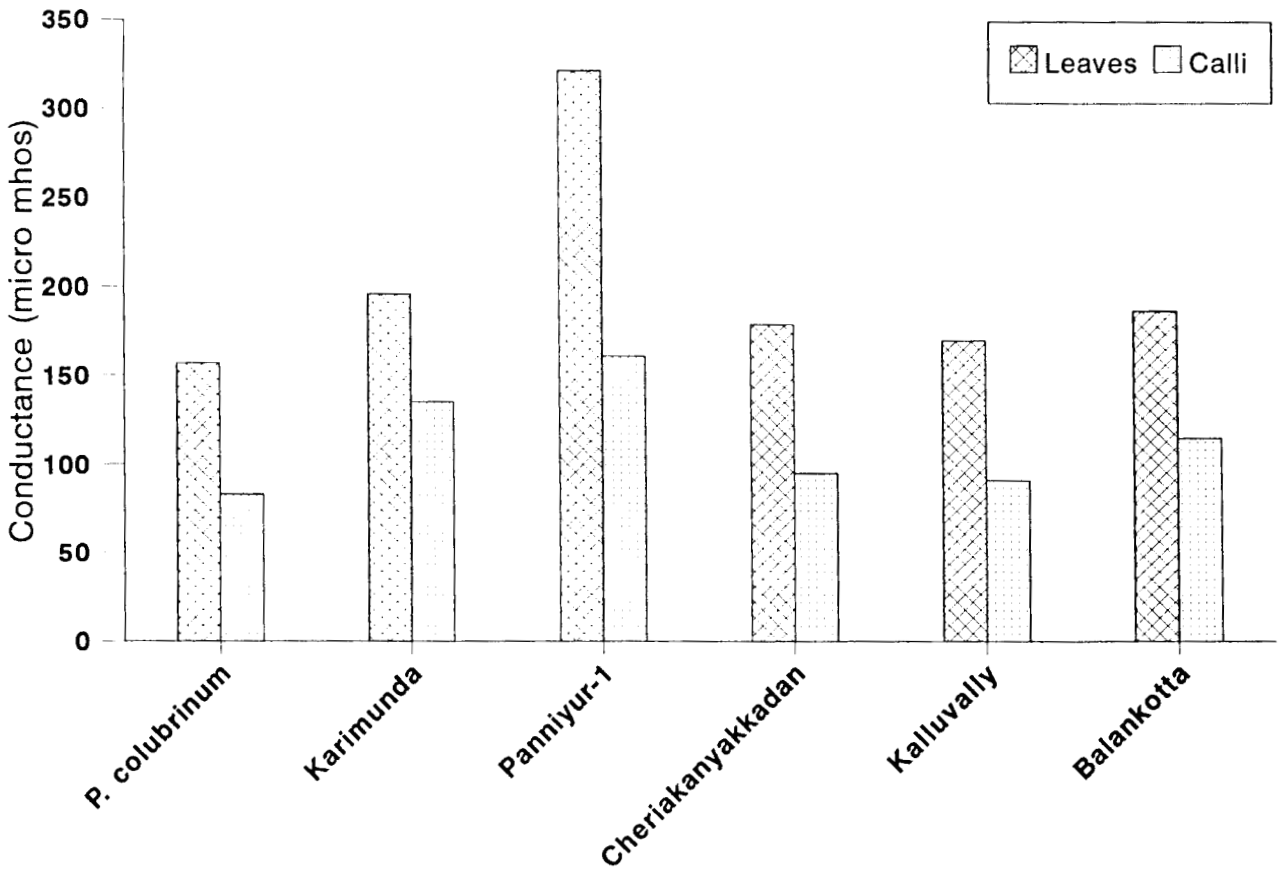
*Average of three replications

Table 6b. Electrolyte leakage induced by concentrated culture filtrate of *Phytophthora capsici* from calli of *Piper nigrum* cultivars and *Piper colubrinum* (calli induced from mature leaf explants)

Genotype	*Leakage of electrolytes 20 minutes after the treatment (μmhos)	*Leakage over control (μmhos)
1. *Piper colubrinum*	116.66	83.33
2. *Piper nigrum*		
a. Panniyur-I	189.33	161.33
b. Karimunda	169.00	135.00
c. Kalluvally	115.66	90.67
d. Cheriakanyakkadan	129.00	95.00
e. Balankotta	143.33	115.33
CD (0.05)		1.6774
SEm $\pm$		0.544

*Average of 3 replications

Fig. 3 Response of pepper genotypes to electrolyte leakage



of *P. colubrinum* (83.33 μmhos) followed by Kalluvally (90.67 μmhos) and Cheriakanyakkadan (95 μmhos). The highest leakage of 161.33 μmhos was exhibited by Panniyur-1 followed by Karimunda (135 μmhos) and Balankotta (115.33 μmhos). Hence the tolerance/resistance observed in the whole plant level of different cultivars was carried as such to the callus phase also.

The calli induced from *in vitro* source of explants of different cultivars exhibited the same pattern in electrolyte leakage and were also found significant (Table 6c). The highest leakage of 179.33 μmhos was recorded by Panniyur-1 followed by Karimunda (128.33 μmhos), Balankotta (101.67 μmhos), Cheria-kanyakadan (83.33 μmhos) and Kalluvally (81.67 μmhos). The tolerance/resistance expressed to *P. capsici* at whole plant level was inherited almost to the same extent in calli induced from *in vitro* raised open pollinated seedlings.

4.1.6 Electrolyte leakage from calli at different dilutions of concentrated culture filtrate

Data pertaining to electrolyte leakage of calli induced from *in vitro* seedling explants at different dilutions of CCF are presented in Table 7.

Electrolyte leakage was induced from calli even at lower concentrations of CCF viz. 2.5-10 per cent v/v and highly significant variation in electrolyte leak-age was observed at different dilutions of CCF irrespective of the cultivars. The lowest leakage of 6.4 μmhos was recorded at 2.5 per cent v/v and the highest leak-age of 26.0 μmhos at the highest concentration of 10 per cent v/v, irrespective of the cultivars. An increasing trend in electrolyte leakage was thus observed at increasing concentrations of CCF.

Table 6c. Electrolyte leakage induced by concentrated culture filtrate of *P. capsici* from black pepper calli
(calli induced from *in vitro* seedling explants)

Cultivars	*Leakage of electrolytes 20 minutes after the treatment (μmhos)	*Leakage over control (μmhos)
1. Panniyur-I	194.33	179.33
2. Karimunda	139.33	128.33
3. Kalluvally	98.66	81.67
4. Cheriakanyakkadan	100.33	83.33
5. Balankotta	121.66	101.67
CD (0.05)		1.562
SEm±		0.494

* Average of three replications

Table 7. Effect of dilution of concentrated culture filtrate on electrolyte leakage from calli of black pepper cultivars

Cultivars	* Electrolyte leakage over control (μmhos) 20 minutes after the treatment at different dilutions of CCF				
	Dilutions of CCF				
	2.5% v/v	5.0% v/v	7.5% v/v	10% v/v	Variety Means
Panniyur-1	7.0	12.5	14.5	61.0	23.75
Cheriakanyakkadan	3.5	7.5	10.0	13.0	8.50
Kalluvally	2.5	7.5	10.0	13.0	8.25
Karimunda	10.5	18.5	23.0	24.0	19.00
Balankotta	8.5	13.5	16.0	19.0	14.25
Concentration means	6.4	11.9	14.7	26.0	
CD (0.05) for comparison of concentration			- 0.722		
CD (0.05) for comparison of cultivars			- 0.807		
CD (0.05) for comparison for interaction			- 1.615		

* Average of two replication

Irrespective of the concentration of CCF, Panniyur-1 exhibited the highest leakage of 23.75 μmhos followed by Karimunda (19.00 μmhos) and Balankotta (14.25 μmhos). However, leakage exhibited by Cheriakanyakkadan and Kalluvally were on par (8.5 and 8.25 μmhos respectively) and were lower than the other cultivars.

In the above studies the variety x concentration interaction effect was also found significant. At all the dilutions of CCF Panniyur-1 and Karimunda significantly differed from each other and from Kalluvally and Cheriakanyakkadan. However, the leakage exhibited by Cheriakanyakkadan and Kalluvally at all the dilutions were on par. So at lower concentrations of toxic metabolite(s) Kalluvally and Cheriakanyakkadan were showing the same type of response.

4.2 *In vitro* screening of calli using concentrated culture filtrate

4.2.1 Standardisation of the level of concentrated culture filtrate for *in vitro* callus screening

Concentrated culture filtrate induced callus necrosis on susceptible calli and it was not possible to revive the necrotic calli in a non toxic medium (Plates 6 to 8).

4.2.1.1 Calli induced from mature leaf segments

The calli induced from mature leaf segments exhibited significant variation in the percentage of necrotic calli at varying concentrations of CCF in the modified MS medium (Table 8a and Fig.4). The highest percentage of callus necrosis was observed in 10 per cent v/v CCF (55.85) followed by 7.5 per cent v/v (43.95), 5.0 per cent v/v (36.50) and 2.5 per cent v/v (30.27).

Plate 6. A small callus bit kept in CCF incorporated medium for screening

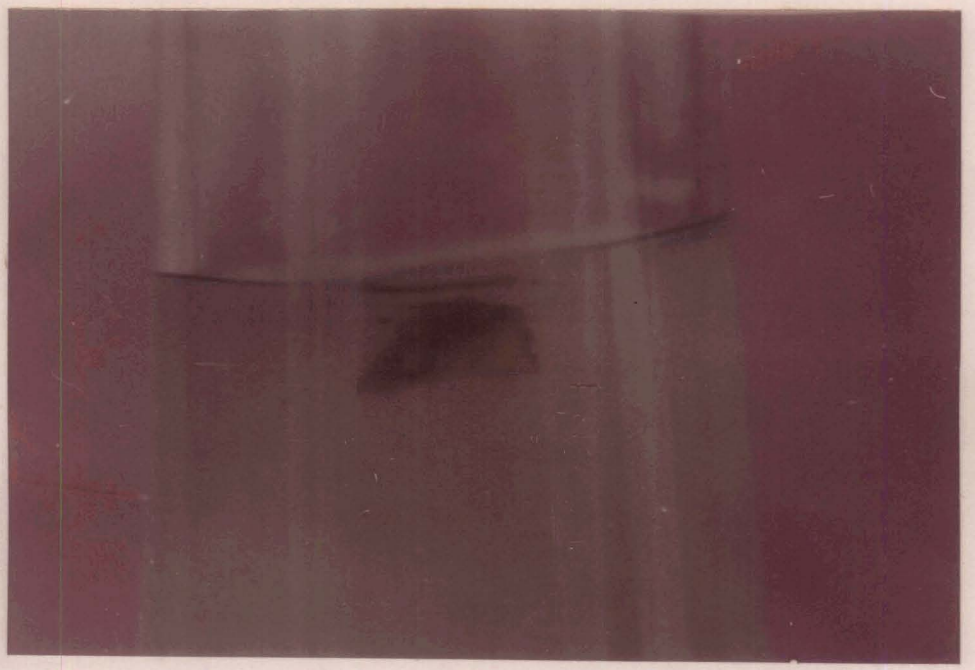


Plate 7. A necrotic callus in CCF incorporated medium

Plate 8. A growing callus in CCF incorporated medium

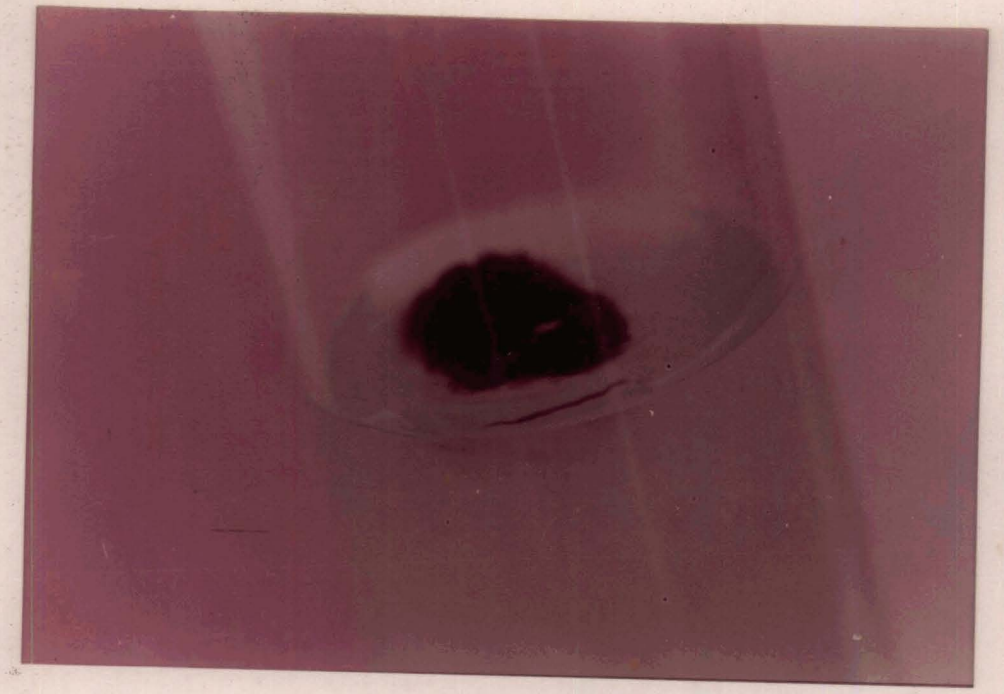


Table 8a. Effect of concentrated culture filtrate on callus necrosis in black pepper cultivars (Calli induced from mature leaf explants)

Cultivars	* Necrotic calli (%)				
	Concentration of CCF in the media				
	2.5% v/v	5.0% v/v	7.5% v/v	10% v/v	Variety means
Kalluvally	16.66 (0.419)**	22.22 (0.478)	27.77 (0.555)	38.88 (0.673)	26.38 (0.531)
Cheriakany-akkadan	18.86 (0.449)	25.00 (0.524)	37.50 (0.662)	46.66 (0.752)	32.01 (0.596)
Balankotta	29.41 (0.568)	36.10 (0.644)	41.17 (0.701)	47.05 (0.764)	38.43 (0.669)
Karimunda	40.00 (0.685)	46.44 (0.750)	53.33 (0.813)	66.66 (0.955)	51.61 (0.801)
Panniyur-1	46.44 (0.752)	52.77 (0.813)	60.00 (0.888)	80.00 (1.120)	59.80 (0.893)
Means	30.27 (0.594)	36.50 (0.642)	43.95 (0.724)	55.85 (0.853)	
CD (0.05) for comparison of varieties			- 0.070		
CD (0.05) for comparison of concentration			- 0.063		
CD (0.05) for comparison of interaction			- 1.409		

Culture period - 3 weeks

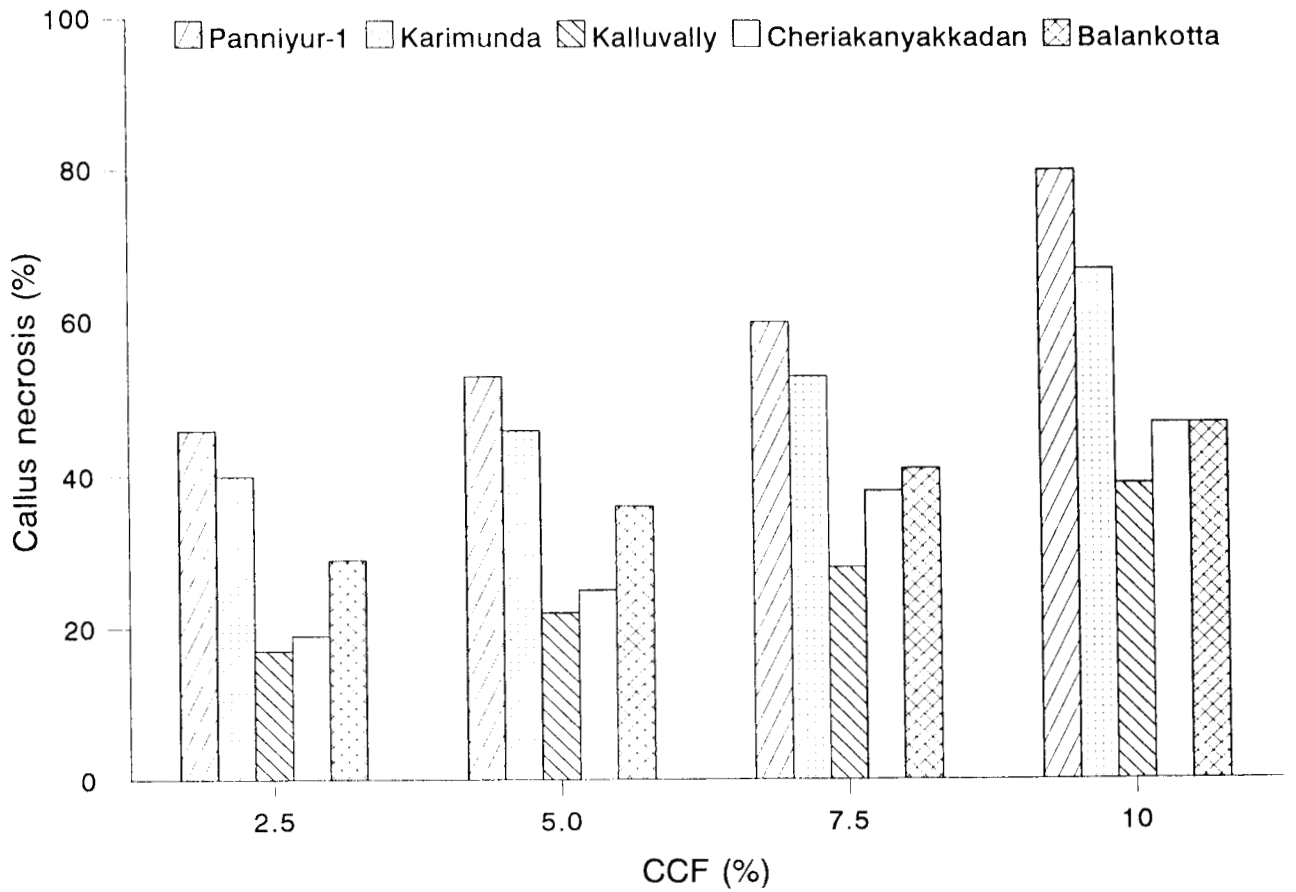
* Average of two replications

** Values in parentheses represent transformed values

The percentage of necrotic calli in medium control at all the levels is 0

Medium - ½ MS supplemented with IAA and BAP 1.0 mg l^{-1}

Fig. 4 Effect of CCF on callus necrosis



The highest percentage of necrotic calli were observed in the cultivar Panniyur-1 (59.80) followed by Karimunda (51.61), Balankotta (38.43), Cheriakanyakkadan (32.01) and Kalluvally (26.38).

However, the variety x concentration interaction effect was not significant. The percentage of necrotic calli in the medium control at all the levels was zero for the different cultivars studied.

Increasing concentration of CCF in the media resulted in increasing percentage of necrotic calli. Since nearly 70 per cent of the calli turned necrotic in the case of cultivars like Panniyur-1 and Karimunda at 10 per cent v/v, the level 7.5 per cent v/v of CCF in the modified MS medium was fixed for screening and studying the effect of CCF at various stages of development.

4.2.1.2 Calli induced from *in vitro* raised seedling explants

The percentage of necrotic calli exhibited at different concentrations of CCF differed significantly (Table 8b). Irrespective of the varieties, the highest percentage of necrotic calli (54.54) was exhibited at CCF 10 per cent v/v in the modified MS medium followed by 7.5 per cent v/v (42.03), 5.0 per cent v/v (33.33) and 2.5 per cent v/v (26.24).

The five cultivars, irrespective of the concentration differed significantly with respect to the percentage of necrotic calli. The highest percentage of necrotic calli was exhibited by Panniyur-1 (53.97) followed by Karimunda (48.32), Balankotta (38.65), Cheriakanyakkadan (31.56) and Kalluvally (22.18).

Table 8b. Effect of concentrated culture filtrate on callus necrosis in black pepper cultivars
(Calli induced from *in vitro* seedling explants)

Cultivars	*Necrotic calli (%)				
	Concentration of CCF in media				
	2.5% v/v	5.0% v/v	7.5% v/v	10% v/v	Variety means
Kalluvally	11.11 (0.340)**	17.64 (0.433)	21.11 (0.477)	38.88 (0.673)	22.18 (0.481)
Cheriakany-akkadan	16.66 (0.419)	27.77 (0.553)	37.03 (0.665)	44.44 (0.730)	31.56 (0.592)
Balankotta	29.16 (0.570)	35.41 (0.637)	43.00 (0.715)	47.05 (0.758)	38.65 (0.670)
Karimunda	35.41 (0.637)	41.42 (0.699)	52.77 (0.813)	64.58 (0.933)	48.32 (0.771)
Panniyur-1	38.88 (0.673)	44.44 (0.730)	54.41 (0.830)	77.77 (1.080)	53.97 (0.828)
Means	26.24 (0.528)	33.33 (0.611)	42.03 (0.700)	54.54 (0.835)	
CD (0.05) for comparison of cultivars			- 0.044		
CD (0.05) for comparison of concentration			- 0.039		
CD (0.05) for comparison of interaction			- 0.087		

Culture period - 3 weeks
* Average of two replications
** Values in parantheses represent transformed values
Percentage of necrtic calli in medium control at all levels is 0
Medium - ½ MS supplemented with IAA and BAP 1.0 mg l^{-1}

In the study, the variety and concentration interaction effect was found to be significant. At lower concentrations of CCF in the media viz. 2.5 and 5.0 per cent v/v Karimunda and Balankotta were on par with respect to percentage of necrotic calli. But at higher concentrations of CCF in the medium viz. 7.5 and 10.0 per cent v/v highly significant variation was observed with regard to the percentage of necrotic calli in the above cultivars. The cultivars Kalluvally differed significantly from Cheriakanyakkadan at two levels of CCF in the media viz. 5.0 and 7.5 per cent v/v. At 10 per cent v/v even though Kalluvally registered a lower callus necrosis compared to Cheriakanyakkadan both of them were on par. The cultivars Kalluvally, Cheriakanyakkadan and Balankotta differed significantly from Panniyur-1 in the percentage of calli exhibiting necrosis at all concentrations of CCF. The percentage of necrotic calli in the medium control at all the levels was found to be zero for the different cultivars studied.

4.2.2 Effect of concentrated culture filtrate on callus growth and proliferation

4.2.2.1 Calli induced from mature leaf explants

The percentage of surviving calli in CCF incorporated medium (7.5% v/v) was found to be low as compared to the control for all the cultivars (Table 9a). However, Kalluvally registered a higher survival percentage of 72.73 followed by Cheriakanyakkadan (62.50), Balankotta (58.83), Karimunda (46.67) and Panniyur-1 (40.00). The percentage of surviving calli in the medium control for all the cultivars was 100 per cent.

As regard to the growth of the surviving calli, there was not much difference observed in CCF added medium and the medium control. The highest

Table 9a. Effect of concentrated culture filtrate (7.5% v/v) on callus growth and proliferation in black pepper cultivars
(Calli induced from mature leaf explants)

Cultivars	Treatments	Surviving calli (%)	Average growth score	Callus growth index
Kalluvally	CCF medium	72.73	2.90	210.91
	Medium control	100.00	2.95	295.00
Cheriakanyakkadan	CCF medium	62.50	2.85	178.12
	Medium control	100.00	2.88	288.00
Balankotta	CCF medium	58.83	2.55	150.00
	Medium control	100.00	2.50	250.00
Karimunda	CCF medium	46.67	3.00	140.01
	Medium control	100.00	3.00	300.00
Panniyur-1	CCF medium	40.00	2.90	116.00
	Medium control	100.00	2.95	295.00
Culture period				3 weeks
$^{6}X^{2}$ values for comparison of callus growth index of cultivars				33.652**

Medium - ½ MS supplemented with IAA and BAp 1.0 mg l^{-1}

callus growth score in CCF incorporated medium was registered by Karimunda (3.00) followed by Kalluvally (2.90) and Panniyur-1 (2.90). The lowest growth score was recorded by Balankotta (2.55) followed by Cheriakanyakkadan (2.88). Once the calli survived in CCF incorporated medium, CCF was not inhibiting further growth of the calli.

The calli grown in the medium control registered significantly higher callus index as compared to those grown in CCF added medium. In both the media the highest callus growth index was reported by Kalluvally followed by Cheriakanyakkadan, Balankotta, Karimunda and Panniyur-1. The medium control registered higher callus growth index (79.61%) compared to CCF added medium irrespective of the varieties.

4.2.2.2 Calli induced from *in vitro* seedling explants

Data relating to the survival and proliferation of calli from *in vitro* seedling explants in CCF incorporated medium is presented in Table 9b. Slightly higher percentage of survival in CCF added medium was observed for calli induced from *in vitro* source of explants as compared to calli from mature leaf segments.

The growth score registered for the surviving calli in CCF added medium was similar to the control. The growth score in CCF added medium was found to be poor in Balankotta (2.00) and Panniyur-1 (2.50) when compared with the calli induced from mature leaf segments where the growth scores were 2.55 and 2.90 respectively.

χ^2 analysis showed that cultivars differed significantly in callus growth index in CCF added medium and medium control. The highest callus growth index

Table 9b. Effect of concentrated culture filtrate (7.5% v/v) on callus growth and proliferation in black pepper cultivars
(Calli induced from *in vitro* seedling explants)

Cultivars	Treatments	Surviving calli (%)	Average growth score	Callus growth index
Kalluvally	CCF medium	78.89	2.87	226.41
	Medium control	100.00	2.85	285.00
Cheriakanyak-kadan	CCF medium	62.63	2.75	172.23
	Medium control	100.00	2.80	280.00
Balankotta	CCF medium	57.00	2.00	114.00
	Medium control	100.00	2.25	225.00
Karimunda	CCF medium	48.40	3.00	145.20
	Medium control	100.00	3.00	300.00
Panniyur-1	CCF medium	45.56	2.50	113.90
	Medium control	100.00	2.50	250.00

Culture period - 3 weeks
χ^2 values for comparison of callus growth index of cultivars - 56.412**
Medium - ½ MS supplemented with IAA and BAP 1.0 mg l^{-1}

in CCF added medium was observed in Kalluvally (226.41) followed by Cheria-kanyakkadan (172.23), Karimunda (145.20), Balankotta (114.00) and Panniyur-1 (113.90). Karimunda registered the highest callus growth index (300) in medium control. Irrespective of the varieties the medium control registered 73.63 per cent higher callus growth index as compared to CCF added medium.

4.3 Effect of concentrated culture filtrate at various stages of development of calli

4.3.1 Effect of prolonged duration of selection with concentrated culture filtrate on shoot regeneration from calli

4.3.1.1 Calli induced from mature leaf explants

The effect of CCF on regeneration of shoots from calli was studied for three subcultures each with a culture period of three weeks. None of the calli of the different cultivars under study put forth shoots in CCF added medium and in medium control.

4.3.1.2 Calli induced from *in vitro* seedling explants

The effect of CCF on induction of shoots from calli of *in vitro* seedling explants is presented in Table 10.

Regeneration of shoots was not observed in any of the cultivars except Karimunda in CCF added medium in the Ist, 2nd and 3rd subcultures. On the other hand medium control registered regeneration of shoots for all the cultivars. Karimunda registered early induction of shoots and gave rise to shoot initials within 3-5 days of inoculation where as the other cultivars gave initials only in the first subculture.

Table 10. Effect of prolonged duration of selection with concentrated culture filtrate on shoot regeneration from black pepper calli
(Calli induced from *in vitro* seedling explants)

Cultivars	Treatments	Regeneration of shoots (%)			
		1st culture	1st subculture	2nd subculture	3rd subculture
Kalluvally	CCF medium	Nil	Nil	Nil	Nil
	Medium control	Nil	55.55	-	-
Cheriakan-yakkadan	CCF medium	Nil	Nil	Nil	
	Medium control	Nil	52.94	-	-
Balankotta	CCF medium	Nil	Nil	Nil	Nil
	Medium control	Nil	50.00	-	-
Karimunda	CCF medium	62.50	-	-	-
	Medium control	64.70	-	-	-
Panniyur-1	CCF medium	Nil	Nil	Nil	Nil
	Medium control	Nil	27.77	-	-

Culture period - 3 weeks in each subculture
Medium - ½ MS supplemented with IAA and BAP 1.0 mg l^{-1}

The shoots of Karimunda regenerated in CCF added medium became weak and chlorotic when continuously subcultured to CCF added modified MS medium and 95 per cent of the cultures could not be carried over to the rooting stage. Out of the 5 per cent of the cultures, the rootable shoots produced when kept for rooting gave rise to weaklings which rot during hardening.

4.3.2 Effect of concentrated culture filtrate on proliferation of shoots

The effect of CCF on further proliferation of shoots in cultivars like Kalluvally, Karimunda, Cheriayakanyakkadan and Balankotta was compared with the control using 't' test and the results are presented in Table 11. Observations recorded one month after inoculation showed that there was no significant difference in the proliferation of shoots between the two media. The proliferation of shoots observed for Kalluvally in CCF added media was 7.25 where as it was 7.16 in the medium control. The highest proliferation of shoots was observed in Kalluvally in both the media. The lowest proliferation of shoots was observed for Karimunda which registered 4.16 shoots in CCF added medium and 4.0 shoots in medium control.

4.3.3 Effect of concentrated culture filtrate on shoot growth

The increment in mean length of shoots and leaf and node production were compared between the CCF added medium and the medium control using 't' test. The data relating to the effect of CCF on the above parameters are presented in Table 12.

Table 11. Effect of concentrated culture filtrate (7.5% v/v) on proliferation of shoots in black pepper cultivars

Cultivars	No. of shoots* proliferated		t value	Probability	S/NS
	CCF medium	Medium control			
Kalluvally	7.25 (2.689)**	7.16 (2.674)	0.2691	0.7903	N.S.
Karimunda	4.16 (2.034)	4.00 (1.992)	0.5631	0.5791	N.S
Cheriakanyakkadan	4.83 (2.191)	4.91 (2.211)	0.2622	0.7956	N.S
Balankotta	5.00 (2.230)	4.83 (2.191)	0.5424	0.5930	N.S

Culture period - 1 month
Container - Small culture tubes (15 x 2.5 cm)
* Average of 12 observations
** Values in parentheses indicate transformed values
Medium - ½ MS supplemented with IAA and BAP 1.0 mg l^{-1}

Table 12. Effect of concentrated culture filtrate (7.5% v/v) on shoot growth in black pepper cultivars

Cultivars	Treatments		*Increment in shoot length	*Increment in number of nodes produced	*Increment in number of leaves produced
Kalluvally	CCF medium		0.750	1.104	1.345
	Medium control		0.725	1.138	1.481
		t value	0.7355	0.4318	1.6081
		Probability	0.4698	0.6701	0.1221
Cheriakany-akkadan	CCF medium		0.617	1.398	1.173
	Medium control		0.650	1.406	1.478
		t value	0.8424	0.1023	3.0731
		Probability	0.4086	0.9195	0.0056
Balankotta	CCF medium		0.325	1.242	1.104
	Medium control		0.300	1.276	1.276
		t value	0.7609	0.4052	2.1589
		Probability	0.4548	0.6893	0.0420
Karimunda	CCF medium		0.342	1.264	1.276
	Medium control		0.358	1.226	1.242
		t value	0.5566	0.4152	0.4052
		Probability	0.5834	0.6824	0.6893

Culture period - 1 month
* Average of 12 observations
Medium - ½ MS supplemented with IAA 0.1 mg l^{-1} and BAP 0.2 mg l^{-1}

With respect to increment in shoot length and increment in node production no significant differences were observed between the CCF added medium and the medium control in all the cultivars studied.

In the case of leaf production, Cheriakanyakkadan and Balankotta recorded significantly higher leaf number in medium control while there was no significant difference in leaf production in other cultivars between the two media studied. Cheriakanyakkadan registered 1.48 leaves in medium control and 1.17 leaves in CCF added medium. Likewise, Balankotta registered 1.28 leaves in medium control while in CCF added medium it registered 1.10 leaves.

4.3.4 Effect of concentrated culture filtrate on root growth

The effect of CCF on length, number and thickness of roots was studied in two cultivars viz. Kalluvally and Cheriakanyakkadan (Table 13 and Plate 9).

The number of roots showed no significant variation in CCF added medium and the medium control.

The length and thickness of roots showed significant variation in CCF added medium and the control for the two cultivars studied.

In the case of Kalluvally the mean root length was 4.06 cm in CCF incorporated medium where as it was only 1.69 cm in the medium control. Similarly in Cheriakanyakkadan the root length in CCF medium was 3.3 cm where as it was 1.98 cm in the medium control.

The roots produced in CCF incorporated medium were thinner in the case of the two cultivars studied. The thickness (diameter) of roots for Kalluvally in

Table 13. Effect of concentrated culture filtrate (7.5% v/v) on root growth in black pepper cultivars

Cultivars	Treatments		*No. of roots	*Length of roots (cm)	*Thickness of roots (mm)
Kalluvally	CCF medium		10.83 (3.275)**	4.058	0.642
	Medium control		10.75 (3.260)	1.692	0.875
		t value	0.1000	19.1394	4.6549
		Probability	0.9213	0.0000	0.0001
Cheriakany-akkadan	CCF medium		15.83 (3.906)	3.300	0.617
	Medium control		14.66 (3.803)	1.983	0.975
		t value	0.3874	8.3315	6.5162
		Probability	0.7022	0.0000	0.0000

Culture period - 40 days
* Average of 12 observations
** Values in parantheses indicate transformed values
Medium - ½ MS supplemented with sucrose (2%) and IBA 1.0 mg l^{-1}

Plate 9. Plantlet of Cheriakanyakkadan with thinner roots in CCF incorporated medium

1. Rooting medium
2. Rooting medium + CCF 7.5% v/v
3. Medium control


1
2
3

CCF incorporated medium was 0.64 mm and it was 0.88 mm in the medium control. For Cheriakanyakkadan the thickness of roots in CCF added medium was 0.62 mm where as in the medium control it was 0.98 mm.

4.4. Direct selection of the calli by the different screening methods

Since there was no regeneration of shoots from the calli when continuously grown in CCF incorporated medium, continuous culture in CCF incorporated medium was abandoned and only direct screening of the calli was resorted to using CCF. The direct screening of the calli was done by three different methods and the effect of screening the calli on the various stages of development are presented in the following sections. Since regeneration was not achieved in calli of mature leaf segments both in the medium control and CCF added medium, only calli from *in vitro* seedling explants were used for direct screening studies.

4.4.1 Method 1 (Screening the calli by growing in concentrated culture filtrate incorporated modified MS medium)

Large scale screening of calli of all the cultivars was done by growing in CCF incorporated (7.5% v/v) modified MS medium. The calli showing necrosis were discarded and the surviving calli were transferred to normal regeneration medium (½ MS supplemented with IAA and BAP 1.0 mg l^{-1}) free of CCF.

4.4.1.1 Effect of callus screening (Method 1) on regeneration of shoots

The percentage of cultures showing regeneration of shoots in Ist subculture of the screened and unscreened calli is presented in Table 14. There was no significant variation in percentage of cultures showing regeneration of shoots from screened and unscreened calli in cultivars like Kalluvally, Balankotta,

Table 14. Effect of callus screening (Method 1) on regeneration of shoots in black pepper cultivars

Cultivars	*Cultures showing regeneration of shoots in first subculture of calli (%)	
	Screened	Unscreened
Kalluvally	55.55 (0.842)**	58.33 (0.856)
Balankotta	50.00 (0.785)	52.94 (0.804)
Cheriakanyakkadan	55.55 (0.842)	55.55 (0.842)
Karimunda	60.00 (0.888)	64.70 (0.932)
Panniyur-1	0.00 (0.116)	30.55*** (0.592)
CD (0.05) for comparison of cultivars		- 0.093
CD (0.05) for comparison of treatment		- 0.058
CD (0.05) for comparison of interation		- 0.131

Culture period - 3 weeks
* Average of three replications
** Values in parentheses indicate transformed values
*** Regeneration in second subculture
Medium - ½ MS supplemented with IAA and BAP 1.0 mg l^{-1}

Cheriakanyakkadan and Karimunda. None of the Panniyur-1 calli grown in CCF incorporated medium exhibited regeneration of shoots even though 30.55 per cent of cultures put forth shoots from unscreened calli.

4.4.1.2 Effect of callus screening (Method 1) on proliferation of shoots

The cultures regenerated from the screened and unscreened calli showed no significant variation with respect to proliferation of shoots in all the cultivars studied (Table 15 and Plates 10a to 10d).

4.4.1.3 Effect of callus screening (Method 1) on the recovery of rootable shoots

The recovery of rootable shoots from screened and unscreened cultures of Kalluvally, Balankotta, Cheriakanyakkadan and Karimunda is presented in Table 16. There was no significant variation in the recovery of rootable shoots in cultures of screened and unscreened calli in the four cultivars studied (Plates 10a to 10d).

4.4.1.4 Effect of callus screening (Method 1) on root growth

Screening of calli was found to influence the root growth of regenerants significantly (Table 17, Fig.5 and Plate 11a and b).

The regenerants produced from screened calli registered significantly lower number of roots compared to regenerants from unscreened calli in cultivars like Kalluvally, Balankotta and Karimunda. However, Cheriyakanyakkadan was not showing such significant difference although the number of roots produced were less in regenerants from screened calli.

Table 15. Effect of callus screening (Method 1) on proliferation of shoots in black pepper cultivars

Cultivars	* No. of shoots emerged (in 2nd subculture)		t value	Probability
	Screened	Unscreened		
Kalluvally	18.74 (4.318)**	18.66 (4.292)	0.1184	0.9068
Cheriakanyakkadan	15.91 (3.931)	15.66 (3.896)	0.1205	0.9052
Balankotta	11.16 (3.322)	11.33 (3.347)	0.1637	0.8715
Karimunda	12.16 (3.485)	12.25 (3.496)	0.1651	0.8704

Culture period - 3 weeks
Container - Big culture tubes 380 x 200 mm)
* Average of 12 observations
** Values in parentheses indicate transformed values
Medium - ½ MS supplemented with IAA and BAP 1.0 mg l^{-1}

Table 16. Effect of callus screening (Method 1) on the recovery of rootable shoots in black pepper cultivars

Cultivars	* No. of rootable shoots (5th subculture)		t value	Probability
	Screened	Unscreened		
Kalluvally	11.00 (3.31)**	11.16 (3.337)	0.3222	0.7503
Balankotta	9.83 (3.127)	9.91 (3.145)	0.2035	0.8406
Cheriakanyakkadan	10.17 (3.181)	10.08 (3.168)	0.1424	0.8881
Karimunda	5.08 (2.250)	5.00 (2.232)	0.3052	0.7631

Culture period - 3 weeks
* Average of 12 observations
** Values in parantheses indicate transformed values
Medium - ½ MS supplemented with IAA 0.1 mg l^{-1} and BAP 0.2 mg l^{-1}

Plate 10. Regenerated cultures from screened and unscreened calli showing no variation in proliferation and recovery of rootable shoots

10a. Kalluvally - Screened (T-KL), unscreened (KL)

10b. Balankotta - Screened (T-BL), unscreened (BL)


KL
T-KL


T-BL
BL

10c. Cheriakanyakkadan - Screened (T-CK), unscreened (CK)

10d. Karimunda - Screened (T-Ka), unscreened (Ka)


T-CK
CK


T-ka
Ka

Table 17. Effect of callus screening (Method 1) on root growth in black pepper cultivars

Cultivar	Particulars	*Root number		*Root length (cm)		*Root thickness (mm)	
		Screened	Unscreened	Screened	Unscreened	Screened	Unscreened
Kalluvally	Mean	11.33 (3.326)**	15.83 (3.947)	2.983	2.750	0.767	1.017
	t value	2.8439		0.7173		4.3138	
	Probability	0.0094		0.4807		0.0003	
Cheriakanya-kkadan	Mean	14.16 (3.694)	17.66 (4.163)	2.817	3.042	0.917	1.267
	t value	1.6810		0.7549		3.7282	
	Probability	0.1069		0.4583		0.0012	
Balankotta	Mean	9.16 (2.993)	13.66 (3.666)	2.783	2.792	0.733	1.083
	t value	3.3694		0.0312		5.9076	
	Probability	0.0028		0.9754		0.0000	
Karimunda	Mean	8.33 (2.881)	12.25 (3.468)	2.625	2.642	0.683	1.117
	t value	3.8190		0.1068		7.5631	
	Probability	0.0009		0.9159		0.0000	

Culture period - 1 month
* Average of 12 observations
** Values in parentheses indicate transformed values
Medium - ½ MS supplemented with 2% sucrose, IBA 1.0 mg l^{-1}

Fig. 5 Effect of callus screening (method 1) on root growth

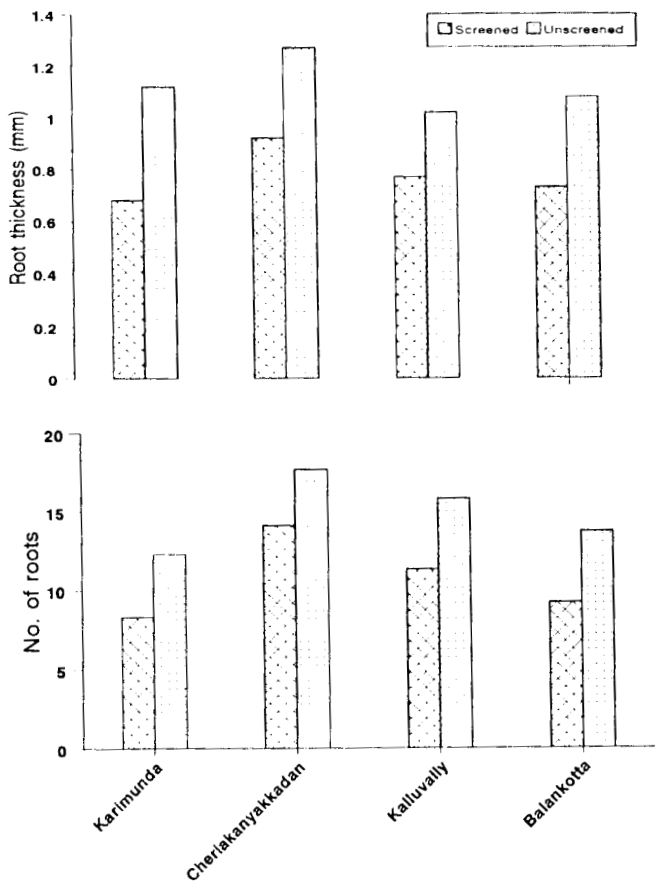


Plate 11. Rooted plantlets regenerated from screened (Method 1) and unscreened calli, with thinner roots in screened calli derived ones

11a. Cheriakanyakkadan - Screened (T-CK), unscreened (CK)

11b. Karimunda - Screened (T-Ka), unscreened (Ka)


CK
T-CK


Ka
T Ka

The mean number of roots recorded in Kalluvally was 11.33 in regenerants from screened calli where as it was 15.83 in regenerants from unscreened calli. Cheriakanyakkadan registered 14.16 roots in regenerants from screened calli and 17.66 in regenerants from unscreened calli. The mean number of roots recorded in plantlets derived from screened calli of Balankotta and Karimunda were 9.16 and 8.33 respectively where as in plantlets derived from unscreened calli the root numbers were 13.66 and 12.25 respectively.

The length of the roots was not found to differ significantly in regenerants from screened and unscreened calli for all the cultivars under study.

The thickness of roots differed significantly in plantlets of screened and unscreened calli. In all the cultivars the roots of the regenerants from the screened calli were found thinner as compared to the roots of the regenerants from unscreened calli. The plantlets from the screened calli of Kalluvally registered a reduced root thickness of 0.77 mm where as the plantlets from unscreened calli recorded significantly greater thickness of 1.02 mm. In the case of Cheriakanyakkadan, Balankotta and Karimunda the thickness of roots in regenerants from screened calli were 0.92 mm, 0.73 mm and 0.68 mm respectively and in regenerants from unscreened calli the thickness were 1.27 mm, 1.08 mm and 1.12 mm respectively.

4.4.1.5 Comparison of the anatomy of roots regenerated from screened and unscreened calli

Comparison of the anatomy of the roots regenerated from screened/ unscreened calli revealed that xylem vessels were not well developed in roots originated from screened calli, the number of layers of cortical cells were less,

cortex was more aerenchymatous and there were more starch grains in cells. In contrast, the roots of the normal regenerants had well developed xylem vessels, more layers of cortical cells, less aerenchyma in the cortex and less starch grains in cells. The roots of the normal regenerants were found to be more thicker due to the presence of more layers of cortical cells when compared to the roots of the screened calli. The variation in diameter of the sections showed the variation in thickness of the roots from the two sources (Plates 12a to 12d).

4.5.2 Method 2 (Screening the calli by shaking in concentrated culture filtrate)

4.5.2.1 Standardisation of the level of concentrated culture filtrate and duration of shaking for screening by method 2

The results of the experiment to standardize the concentration of CCF in the modified MS liquid medium and duration of shaking the calli bits are presented in Table 18.

All the treatments differed significantly from each other. In all the cultivars, the highest percentage of necrotic calli was observed in T_4 (calli shaken for 72 h in 15% v/v CCF added modified liquid MS) followed by T_2 (calli shaken in 15% v/v CCF added liquid MS for 48 h), T_3 (calli shaken for 72 h in CCF added modified liquid MS) and T_1 (calli shaken in 10% v/v CCF added liquid MS for 48 h)

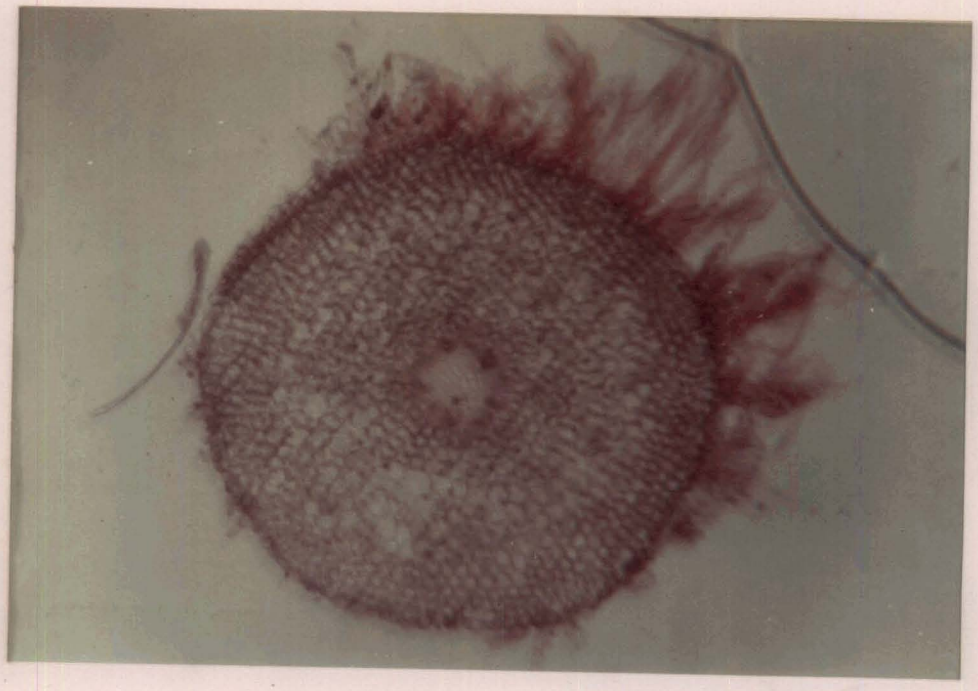
Among the cultivars, Kalluvally registered the lowest callus necrosis followed by Cheriakanyakkadan, Balankotta, Karimunda and Panniyur-1.

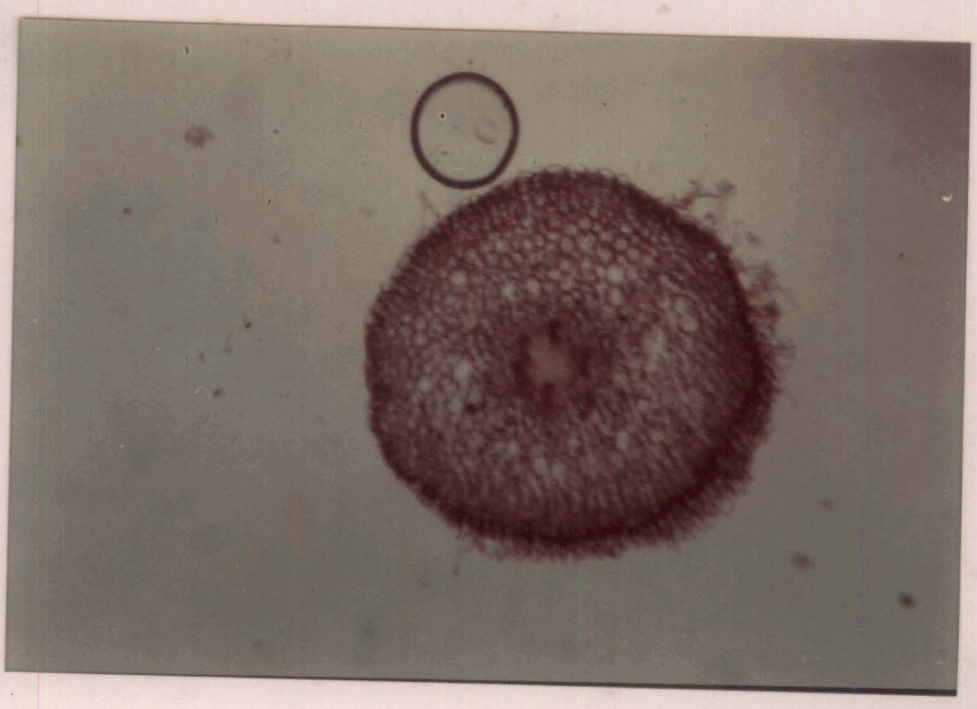
Variety x treatment interaction effect was found to be significant. Even in control shaking when calli were shaken for longer periods callus necrosis was

Plate 12. Comparison of anatomy of roots in plantlets regenerated from screened (Method 1) and unscreened calli

12a. T.S. of root of unscreened calli derived plantlet
Magnification - (x 100)

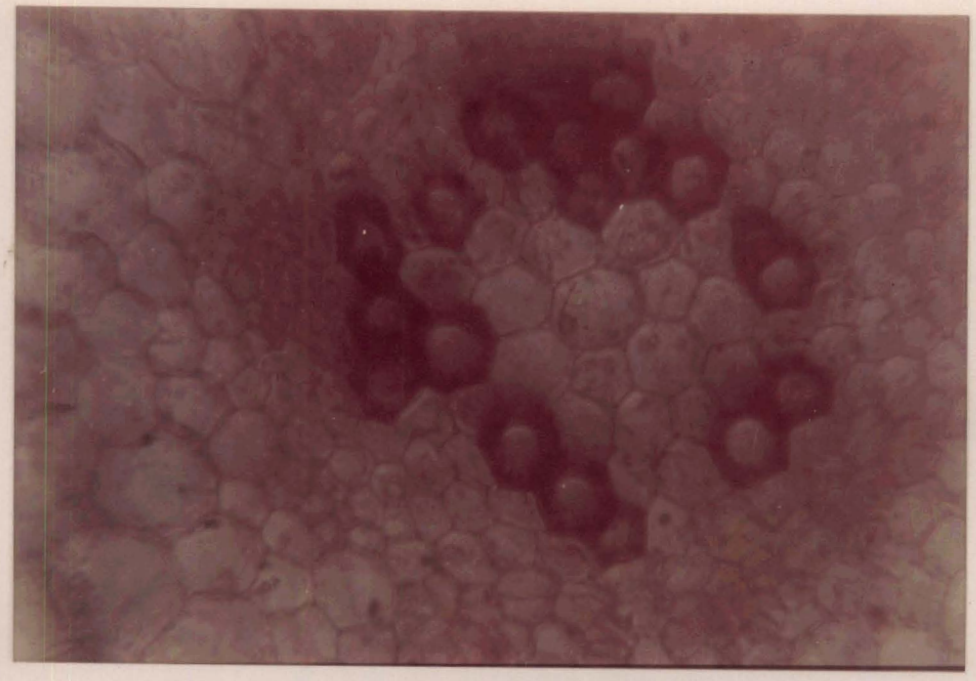
12b. TS of root of Screened calli derived plantlet
Magnification - (x 100)





12c. Well developed xylem vessels - in roots of unscreened calli derived plant
Magnification - (x 1500)

12d. Poorly developed xylem vessels - in roots of screened calli derived plantlet
Magnification - (x 1500)



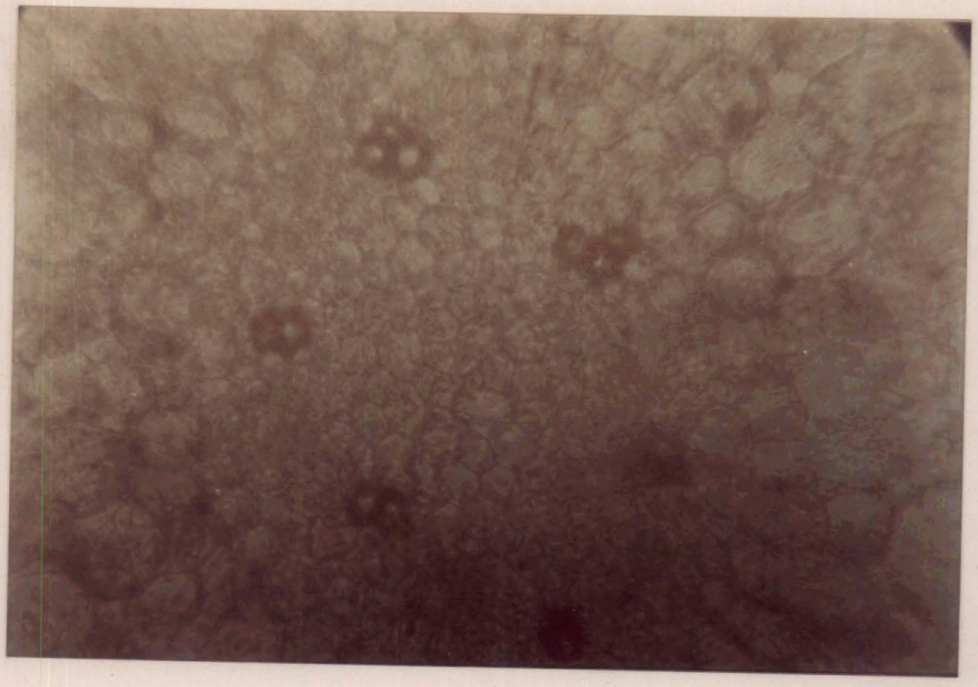


Table 18. Effect of callus screening (Method 2) on callus necrosis in black pepper cultivars

Treatments	*Necrotic calli (%)				
	Kalluvally	Cheriaman-yakkadan	Balankotta	Karimunda	Panniyur-1
T_1 Modified liquid MS + 10% v/v CCF shaking for 48 h	10.00 (0.322)**	19.05 (0.456)	33.33 (0.615)	40.00 (0.685)	47.05 (0.758)
T_2 Modified liquid MS + 15% v/v CCF shaking for 48 h	47.05 (0.758)	50.00 (0.785)	55.55 (0.842)	72.22 (1.018)	83.33 (1.155)
T_3 Modified liquid MS + 10% v/v CCF shaking for 72 h	27.77 (0.555)	33.33 (0.610)	44.44 (0.730)	55.55 (0.842)	77.77 (1.080)
T_4 Modified liquid MS + 15% v/v CCF shaking for 72 h	66.66 (0.955)	77.77 (1.093)	83.33 (1.155)	94.44 (1.333)	100.00 (1.455)
T_5 Modified liquid MS + 10% v/v ***CRM shaking for 48 h	0.00 (0.116)	0.00 (0.116)	0.00 (0.116)	0.00 (0.460)	0.00 (0.116)
T_6 Modified liquid MS + 15% v/v CRM shaking for 48 h	0.00 (0.238)	0.00 (0.116)	0.00 (0.116)	11.11 (0.340)	11.11 (0.340)
T_7 Modified liquid MS + 10% v/v CRM shaking 72 h	5.55 (0.238)	5.55 (0.238)	11.11 (0.340)	16.66 (0.406)	20.00 (0.451)
T_8 Modified liquid MS + 15% v/v CRM shaking for 72 h	11.11 (0.340)	16.66 (0.406)	22.22 (0.491)	27.77 (0.553)	33.33 (0.610)

CD (0.05) for comparison of treatments - 0.075
CD (0.05) for comparison of varieties - 0.059
CD (0.05) for comparison of interaction - 0.167

* Average of two replications
** Values in parentheses indicate transformed values
*** CRM - Concentrated Ribeiro's medium

observed. Higher concentration of CCF in liquid media and longer duration of shaking was found to produce higher percentage of necrotic calli (66.66-100%). So shaking in CCF at 10 per cent v/v for 48 h was followed for screening the calli in further studies.

4.5.2.2.1 Effect of callus screening (Method 2) on shoot regeneration and survival of healthy cultures

The surviving calli after screening by Method 2 were transferred to normal regeneration medium (½ MS supplemented with IAA and BAP 1.0 mg l^{-1}) free of CCF. The callus pieces when transferred to normal regeneration medium put forth friable calli, the regeneration from which was found to be low. All the cultivars registered very low percentage of regeneration of shoots in both the treatments while none of the cultures gave rise to shoot initials in Panniyur-1 (Table 19).

The survival of healthy cultures in the 5th subculture showed that out of the four cultivars showing regeneration of shoots, cultures of Kalluvally alone was found healthy (Plate 13). All the cultures from other cultivars became weak and chlorotic in subsequent subcultures.

4.5.2.2.2 Effect of callus screening (Method 2) on proliferation of shoots and recovery of rootable shoots

The cultures of Kalluvally regenerated from screened calli showed no significant difference in proliferation of shoots as compared to cultures shaken in medium control (Table 20).

Table 19. Effect of callus screening (Method 2) on shoot regeneration and survival of healthy cultures

Cultivar	Treatments	*Regeneration of shoots in 2nd subculture (%)	*Survival of healthy cultures in 5th subculture (%)
Cheriakanyakkadan	CCF shaking	5.78	0
	Control shaking	11.11	0
Balankotta	CCF shaking	7.00	0
	Control shaking	16.66	0
Kalluvally	CCF shaking	5.28	20.00
	Control shaking	12.00	10.00
Karimunda	CCF shaking	13.00	0
	Control shaking	50.00	0
Panniyur-1	CCF shaking	0	0
	Control shaking	0	0

Culture period - 3 weeks in each subculture
* Average of two replications

Table 20. Effect of callus screening (Method 2) on proliferation of shoots and recovery of rootable shoots (cv. Kalluvally)

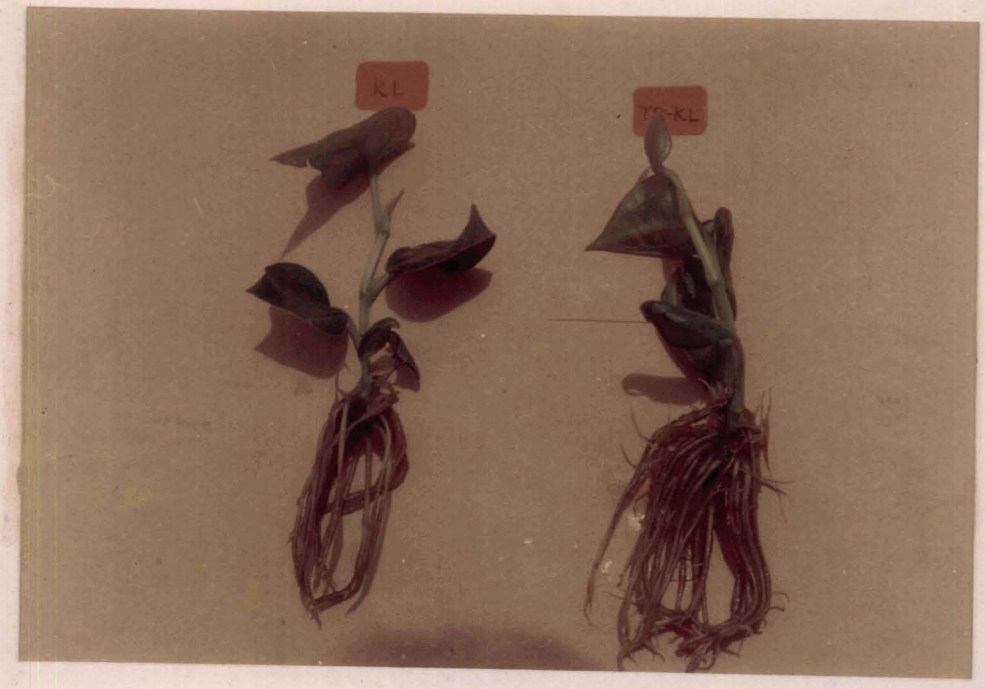
Parameters	Particulars	Treatments	
		CCF shaking	Control shaking
Shoot proliveration* (3rd subculture)	No. of shoots proliferated	7.58 (2.658)	7.83 (2.730)
	t value	0.2510	
	Probability	0.8041	
Recovery of rootable* shoots (6th subculture)	No. of rootable shoots	3.41 (1.802)	3.58 (1.875)
	t value	0.4956	
	Probability	0.6251	

Culture period - 3 weeks
* Average of 12 observations

Plate 13. A culture of Kalluvally regenerated from screened calli by method 2

Plate 14. Rooted plantlets derived from secreened (Method 2) and unscreened calli
Screened (TSKL), unscreened (KL)


TS


KL
TS-KL

4.5.2.2.3 Effect of callus screening (Method 2) on root growth

The root characters in the regenerants from screened calli by CCF shaking were compared with that from control shaken cultures. The length of roots, number of roots and thickness of roots did not differ significantly in screened and unscreened cultures (Table 21 and Plate 14).

4.5.3 Method 3 (Screening the calli by double layer culture technique)

4.5.3.1 Effect of callus screening (Method 3) on callus necrosis and regeneration

The performance of different cultivars in screening by double layer culture technique is presented in Table 22. The double layer with fungus (DLF) significantly differed from control double layer (CDL) in the percentage of necrotic calli in all the cultivars studied except Balankotta (Plate 15). The highest percentage of callus necrosis in double layer with fungus was exhibited by Panniyur-1 (95%) followed by Karimunda (45%), Balankotta (40%), Cheriakanyakkadan (30%) and Kalluvally (25%).

The variety x treatment interaction was found to be significant. The surviving calli when inoculated to modified MS medium supplemented with IAA and BAP 1.0 mg l^{-1}, regeneration of shoots was observed in cultivars like Kalluvally, Cheriakanyakkadan and Karimunda in the Ist subculture. Only the cultures of Kalluvally could be carried over to the final stage of rooting and planting out.

4.5.3.2 Effect of callus screening (Method 3) on proliferation of shoots and recovery of rootable shoots

Comparison of the cultures screened by DLC technique and unscreened culture showed that there was no significant difference with respect to proliferation of shoots and recovery of rootable shoots (Table 23 and Plate 16).

Table 21. Effect of callus screening (Method 2) on root growth (cv. Kalluvally)

Parameters		Treatments	
		Screened	Unscreened
Length of roots* (cm)		2.65	2.625
	t value	0.3452	
	Probability	0.7333	
No. of roots*		12.40 (3.470)**	12.25 (3.488)
	t value	0.0152	
	Probability	0.9888	
Thickness of roots* (mm)		0.975	0.967
	t value	0.2664	
	Probability	0.7924	

Culture period - 1 month
* Average of 12 observations
** Values in parantheses represent transformed values

Table 22. Effect of callus screening (Method 3) on callus necrosis and regeneration in black pepper cultivars

Cultivars	* Necotic calli (%)		* Regenerating calli in 1st subculture (%)		* Survival of healthy cultures in 5th subculture (%)	
	DLF	CDL	DLF	CDL	DLF	CDL
Kalluvally	28 (0.522)**	10 (0.322)	40	0	30	0
Cheriakany-akkadan	30 (0.574)	0 (0.159)	13.33	0	0	0
Panniyur-1	95 (1.410	10 (0.332)	0	0	0	0
Balankotta	40 (0.685)	30 (0.574)	0	0	0	0
Karimunda	45 (0.735)	10 (0.322)	20	0	0	0

CD (0.05) for comparison of varieties - 0.174
CD (0.05) for comparison of treatments - 0.110
CD (0.05) for comparison of interaction - 0.249

Culture period - 3 weeks in each subculture
* Average of two replications
** Values in parantheses indicate transformed values

DLF - Double layer with fungus
CDL - Control double layer

Plate 15. Comparison of callus necrosis in double layer culture of Panniyur-1 and Kalluvally

Left - Panniyur-1 with more number of necrotic calli

Right - Kalluvally with more number of surviving calli

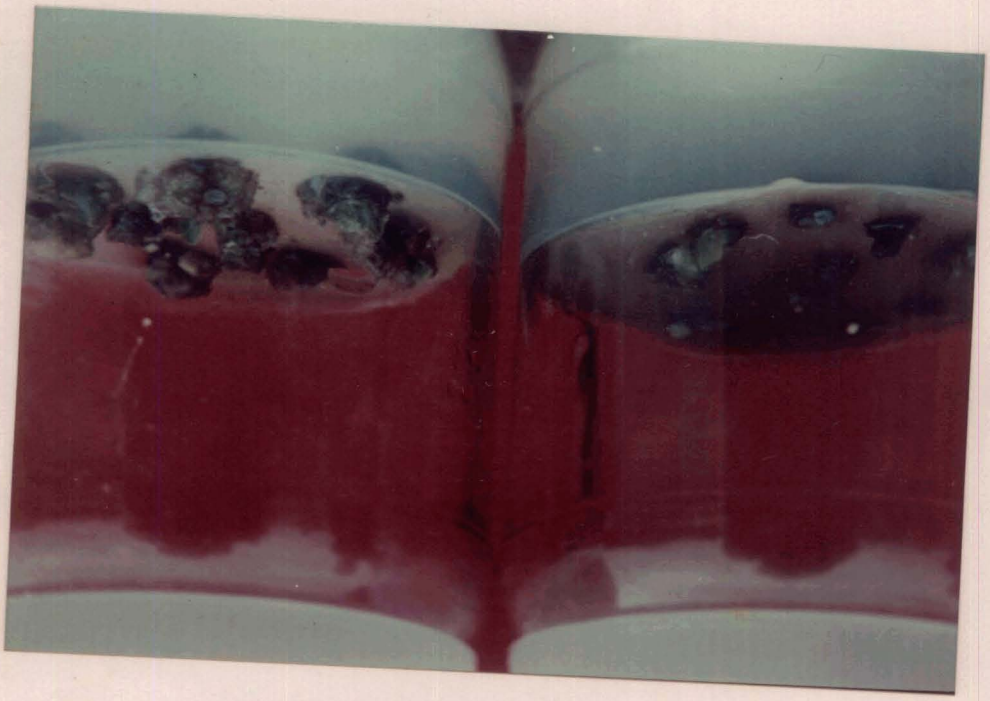


Table 23. Effect of callus screening (Method 3) on proliferation of shoots and recovery of rootable shoots (cv. Kalluvally)

Parameters	Particulars	Treatments	
		Screened	Unscreened
Shoot proliferation * (2nd subculture)	No. of shoots proliferated	18.08 (4.242)**	18.16 (4.235)
	t value	0.0368	
	Probability	0.9710	
Recovery of* rootable shoots (5th subculture)	No. of rootable shoots	11.25 (3.350)	12.00 (3.458)
	t value	1.3572	
	Probability	0.1885	

Culture period - 3 weeks
* Average of 12 observations
** Values in parantheses represent transformed values

4.5.3.3 Effect of callus screening (Method 3) on root growth

The different root characters such as the number of roots, length of roots and thickness of roots were compared in screened and unscreened cultures of the cultivar Kalluvally. These parameters were not found to differ significantly in screened and unscreened cultures (Table 24 and Plate 17).

4.6 *In vitro* induction of mutation using gamma irradiation

4.6.1 Effect of gamma irradiation on callus growth (variety Panniyur-2)

At lower doses of gamma irradiation ranging 2.5 Gy to 15 Gy more than 50 per cent of calli put forth good growth giving higher callus index (Table 25). The higher doses tried viz. 20.0, 50.0 and 100.0 Gy were found to be deleterious for callus growth and only 11 to 25 per cent of calli put forth growth giving a lower callus index. The calli inoculated without subjecting to gamma irradiation registered highest growth and highest callus index.

4.6.2 Effect of gamma irradiation on callus growth (cv. Kalluvally)

Calli induced from mature leaf segments of Kalluvally could withstand higher doses of gamma irradiation compared to the calli induced from Panniyur-2. Kalluvally could withstand gamma irradiation up to 25.0 Gy giving a callus index of 222.20 (Table 26). Compared to the non- irradiated control, the irradiated calli in doses ranging from 17.5 to 25 Gy were found to put forth good growth giving higher callus index.

Table 24. Effect of callus screening (Method 3) on root growth (cv. Kalluvally)

Parameters		Treatments	
		Screened	Unscreened
No. of roots*		14.91 (3.826)**	15.83 (3.947)
	t value	0.5501	
	Probability	0.5878	
Length of roots* (cm)		2.25	2.75
	t value	2.4122	
	Probability	0.0246	
Thickness of roots* (mm)		0.95	1.017
	t value	1.0979	
	Probability	0.2841	

Culture period - 1 month
* Average of 12 observations
** Values in parantheses represent transformed values

Plate 16. Regenerated culture of Kalluvally from screened (Method 3) and unscreened calli
Screened (DLKL), unscreened (KL)

Plate 17. Rooted plantlets derived from screened (Method 3) and unscreened calli
Screened (DLKL), unscreened (KL)


KL
DL KL


KL
DL KL

Table 25. Effect of gamma irradiation on callus growth (var. Panniyur-2)

Dose Gy	Calli putting forth growth (%)	Growth score	Callus growth index
0 (Control)	100.00	2.75	275.00
2.5	87.50	2.42	212.50
5.0	75.00	3.00	225.00
7.5	55.55	3.00	166.65
10.0	75.00	3.00	225.00
15.0	57.14	3.00	171.43
20.0	25.00	1.00	25.00
50.0	16.66	1.00	16.66
100.0	11.11	1.00	11.11

Culture period - 3 weeks
Medium - ½ MS supplemented with IAA and BAP 1.0 mg l^{-1}

Table 26. Effect of gamma irradiation on callus growth (cv. Kalluvally)

Dose Gy	Calli putting forth growth(%)	Growth score	Callus growth index
0 (Control)	87.50	2.28	200.00
15.0	75.00	2.00	150.00
17.5	100.00	2.66	266.00
20.0	100.00	2.42	242.00
22.5	100.00	3.00	300.00
25.0	88.88	2.50	222.20

Culture period - 3 weeks
Medium - ½ MS supplemented with IAA and BAP 1.0 mg l^{-1}

4.6.3 Effect of gamma irradiation on callus growth and regeneration of shoots in black pepper cultivars (Calli induced from mature leaf explants)

There was no significant difference in percentage of calli putting forth growth in the different cultivars at the various irradiation doses tried. The callus growth of the irradiated and non-irradiated cultures when compared, it was found that non-irradiated control recorded the highest growth and significantly differed from all other cultivars (Table 27).

The growth of calli and the nature of calli formed were found to be different at different doses tried. At higher doses (40 and 50 Gy) all the cultivars were producing brown friable calli. In contrast, Kalluvally could withstand a higher dose of gamma irradiation as compared to other cultivars, giving cream nodular calli even at 40.0 Gy. The growth score and callus growth index were also found to be less at higher irradiation doses of 40 and 50 Gy for all the cultivars except Karimunda which recorded better growth score (2.8 and 2.12) and callus index (224 and 212) at higher irradiation doses. The percentage of shoot regeneration was found to be nil for all the cultivars. Evaluating the growth performance, the dose 30.0 Gy was fixed as the maximum limit of gamma irradiation that the calluses of different cultivars could withstand.

4.6.4 Effect of gamma irradiation (30.0 Gy) on callus growth and regeneration (calli induced from *in vitro* seedling explants)

When the calli induced from *in vitro* seedling explants were subjected to gamma irradiation, all the cultures put forth growth either as nodular or friable calli (Table 28). The major portion of the cultures of Cheriakanyakkadan and Karimunda produced friable calli which were found to be non-responding with respect to

Table 27. Effect of gamma irradiation on callus growth and shoot regeneration in black pepper cultivars (calli induced from mature leaf explants)

Cultivars	Dose Gy	Cultures putting forth growth(%)	Growth score	Callus growth index	Nature of calli	Shoot regeneration(%)
Panniyur-2	0 (Control)	100.00(1.412)**	2.75	275.00	CN	Nil
	10.0	75.00(1.049)	2.66	199.50	CN	Nil
	20.0	50.00(0.785)	2.50	125.00	CN	Nil
	30.0	75.00(0.049)	2.83	212.50	CN	Nil
	40.0	86.10(1.191)	1.86	160.15	BF	Nil
	50.0	77.77(1.093)	1.42	110.43	BF	Nil
Kalluvally	0 (Control)	86.10(1.191)	2.71	233.31	CN	Nil
	10.0	60.00(0.888)	3.00	180.00	CN	Nil
	20.0	77.77(1.093)	2.00	155.54	CN	Nil
	30.0	50.00(0.785)	2.50	125.00	CN	Nil
	40.0	77.77(1.093)	2.71	211.09	CN	Nil
	50.0	33.33(0.785)	1.66	55.55	LBF	Nil
Panniyur-1	0 (Control)	86.10(1.911)	2.71	233.31	CN	Nil
	10.0	57.77(0.864)	2.50	144.42	CN	Nil
	20.0	57.77(0.864)	1.50	86.65	CN	Nil
	30.0	88.88(1.246)	1.87	166.65	CN	Nil
	40.0	66.66(0.960)	1.83	122.21	LBF	Nil
	50.0	55.55(0.842)	1.80	99.99	LBF	Nil
Karimunda	0 (Control)	100.00(1.412)	3.00	300.00	CN	Nil
	10.0	77.77(1.093)	3.00	233.31	CN	Nil
	20.0	100.00(1.412)	2.50	250.00	CN	Nil
	30.0	100.00(1.412)	2.33	233.00	CN	Nil
	40.0	80.00(1.120)	2.80	224.00	LBF	Nil
	50.0	100.00(1.412)	2.12	212.00	LBF	Nil

CD (0.05) comparison of varieties - 0.106
CD (0.05) comparison of irradiation dose - 0.234
CD (0.05) comparison of interaction - 0.260

* Average of 2 replications
** Values in parantheses represent transformed values
Medium - ½ MS supplemented with IAA and BAP 1.0 mg l^{-1}
CN - Cream nodular
BF - Brown friable
LBF - Light brown friable

Table 28. Effect of gamma irradiation (30 Gy) on callus growth and shoot regeneration (Calli induced from *in vitro* seedling explants)

Cultivar	Cultures putting forth growth (%)	Nature of calli	Regeneration (%) and nature of shoots
Cheriakanyakkadan	100	CN, CF, BF	Nil
Karimunda	100	CN, LBF, CF	Nil
Kalluvally	100	CN, LBF, BN	6.66 (weak chlorotic shoots)
Balankotta	100	CN, BN, CF	Nil

Medium - ½ MS supplemented with IAA and BAP 1.0 mg l^{-1}

CN - Cream nodular
CF - Cream friable
BF - Brown friable
LBF - Light brown friable
BN - Brown nodular

regeneration. However, regeneration could be achieved in Kalluvally, where weak chlorotic shoots emerged in 6.66 per cent of the cultures which could not be carried over to subsequent subcultures.

4.6.5 *In vitro* screening of the irradiated calli using concentrated culture filtrate

The growth and regeneration of irradiated calli in CCF incorporated modified MS medium is presented in Table 29.

When the irradiated calli at 30.0 Gy were subjected to screening by growing them in CCF incorporated modified MS medium, few calli (5.00-27.77%) put forth growth in CCF added medium for cultivars like Karimunda, Kalluvally and Balankotta. But Cheriakanyakadan registered better growth giving 55.55 to 60.00 per cent growth of calli. However, regeneration could not be achieved in any of the cultivars except Kalluvally where 5.55 per cent of cultures put forth very weak shoots and could not be carried over to subsequent subcultures.

4.7 Partial purification of the culture filtrate of *Phytophthora capsici*

4.7.1 Separation of the toxic metabolite(s) from concentrated culture filtrate by organic solvent fractionation

The aqueous and the solvent fractions of CCF in methanol, acetone, dichloro ethane, diethyl ether, chloroform and ethyl acetate were separately collected. Uniform drops of these fractions were assayed using leaf puncture bioassay in detached leaves of the cultivar Karimunda. The development of symptoms 60 h after inoculation is presented in Table 30. Symptoms were found to produce only in aqueous fractions showing that the toxic metabolite(s) could not be separated using the organic solvents.

Table 29. Effect of screening the irradiated calli on callus growth and regeneration (Calli induced from *in vitro* seedling explants)

Cultivars	Treatment	Calli putting forth growth(%)	Shoot regeneration (%) and nature of shoots
Cheriakanyakkadan	CCF 7.5% v/v	55.55	Nil
	Medium control	60.00	Nil
	CCF 10% v/v	60.00	Nil
	Medium control	80.00	Nil
Karimunda	CCF 7.5% v/v	12.50	Nil
	Medium control	87.50	Nil
	CCF 10% v/v	12.50	Nil
	Medium control	37.50	Nil
Kalluvally	CCF 7.5% v/v	25.00	5.55 (Very weak shoots)
	Medium control	25.00	Nil
	CCF 10% v/v	27.77	Nil
	Medium control	37.50	Nil
Balankotta	CCF 7.5% v/v	25.00	Nil
	Medium control	55.55	Nil
	CCF 10% v/v	5.00	Nil
	Medium control	37.50	Nil

Medium - ½ MS supplemented with IAA and BAP 1.0 mg l^{-1}

Table 30. Effect of organic solvent fractionation for separation of toxic metabolite(s) from culture filtrate of *Phytophthora capsici*

Solvent	Fraction	Symptom development	Average lesion* diameter in cm
Methanol	Methanol	-	-
	aqueous	+	1.0
Ethylacetate	Ethylacetate	-	-
	aqueous	+	1.25
Chloroform	Chloroform	-	-
	aqueous	+	2.0
Ether	Ether	-	-
	aqueous	+	2.5
Dichloromethane	Dichloromethane	-	-
	aqueous	+	2.0
Acetone	Acetone	-	-
	aqueous	+	2.75

* average of 4 replications
\+ indicates development of symptoms

4.7.2 Separation of the toxic metabolite(s) from concentrated culture filtrate by ion exchange chromatography

Out of the 13 fractions collected from Dowex 1, typical symptoms were observed in 7th, 8th and 9th fractions as evident by the leaf puncture bioassay. Further purifications and concentration of these fractions could be effected in Dowex 50. Leaf puncture bioassay of the 18 fractions collected from Dowex 50 showed typical symptoms in 7th, 8th and 9th fractions. The intensity of lesion development in fractions collected from Dowex 50 was more than that from Dowex 1 which was due to the concentration effect in Dowex 50 column (Plate 18).

4.7.3 Analysing the concentrated culture filtrate for the presence of coumarin

Thin layer chromatography analysis was carried out to detect the presence of coumarin in culture filtrate of *P. capsici*. When coumarin standard was spotted and kept in iodine chamber typical spot of coumarin was developed. Concentrated culture filtrate when spotted in silica plate and kept in iodine chamber, no spot of coumarin was found to develop. The results of the analysis showed that coumarin was not present in the concentrated culture filtrate.

Leaf puncture bioassay was done with coumarin standard. Since coumarin was found to be insoluble in water it was dissolved in small quantity of ethyl alcohol and the volume made up with sterile distilled water. This solution when bioassayed no symptoms were observed on detached leaves. The result showed that the toxic metabolite(s) were not coming in the coumarin group.

The aqueous fractions after separating the gummy substance for TLC analysis was bioassayed. Typical symptoms of *P. capsici* were produced in the bioassay indicating the presence of toxic principles in the aqueous fraction. Even

Plate 18. Comparison of symptoms induced by CCF and fractions from DW 1 and DW 50


C.C.F
D+W I
D+W 50

after hydrolysis, diethyl ether could not separate the toxic principles and hence the toxic metabolite(s) present in the culture filtrate were totally in the aqueous fraction.

4.8 Production of somaclones without *in vitro* callus screening and screening the regenerants at whole plant level

4.8.1 Induction of calli

4.8.1.1 Response of black pepper cultivars to callus induction and proliferation

4.8.1.1.1 Calli induced from mature leaf segments

Cultivars differed significantly in the days taken for callusing, percentage of callusing and callus proliferation (Table 31a and Plate 19). Cent per cent callusing was observed in cultivars like Kalluvally and Balankotta. The percentage of callusing exhibited by Karimunda (92.96), Panniyur-1 (85.92), Cheriakanyakkadan (78.88) and *P. colubrinum* (80.00) significantly differed from that of Kalluvally and Balankotta. However, the percentage of callusing exhibited by Cheriakanyakkadan, Panniyur-1 and *P. colubrinum* were on par.

The highest growth score of 3.00 was registered by Balankotta followed by Kalluvally (2.73) and Karimunda (2.37). The lowest growth score was recorded by Panniyur-1 (1.66) followed by *P. colubrinum* (2.00) and Cheriakanyakkadan (2.05).

The highest callus index (300) was observed for Balankotta followed by Kalluvally (273) and Karimunda (220.31).

In all the cultivars callusing was initiated in 10.33 to 13 days. Early callusing was observed in Kalluvally followed by Panniyur-1 (11.00) which differed significantly from late callusing type like Karimunda (12.66) and *Piper colubrinum* (13.00).

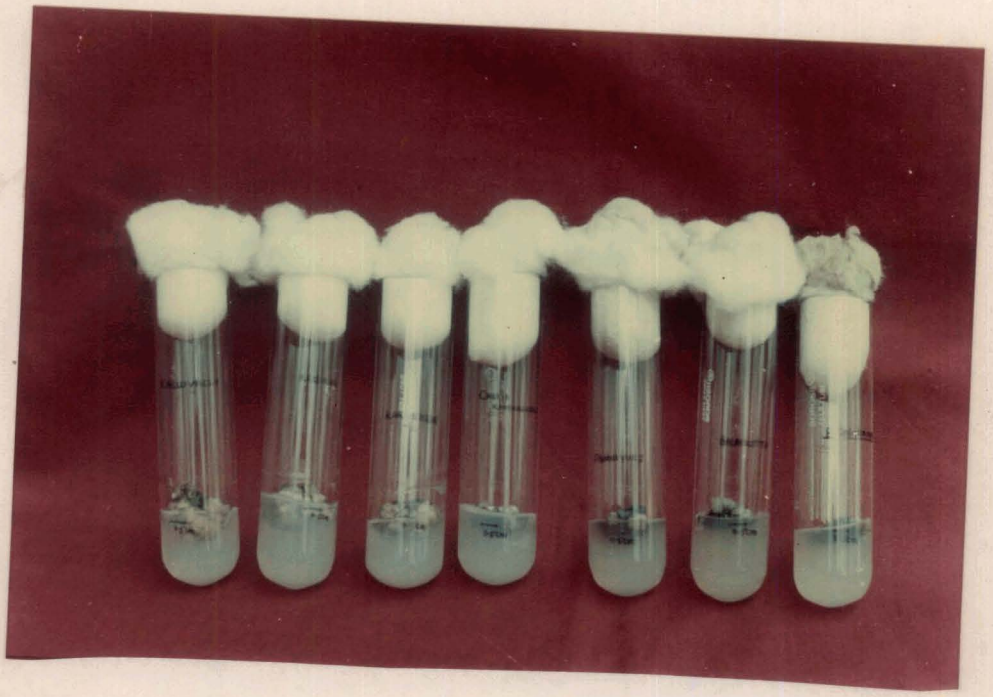
Table 31a. Response of pepper genotypes to callusing and callus growth in black pepper (Calli induced from mature leaf explants)

Genotype	*Callusing (%)	*Growth score	Callus index	*Days for callus initiation
Panniyur-1	85.92 (1.190)**	1.66	142.62	11.00 (3.314)
Karimunda	92.96 (1.312)	2.37	220.31	12.66 (3.558)
Cheriakanyakkadan	78.88 (1.101)	2.05	161.70	12.00 (3.462)
Kalluvally	100.00 (1.455)	2.73	273.00	10.33 (3.214)
Balankotta	100.00 (1.455)	3.00	300.00	12.33 (3.511)
P. colubrinum	80.00 (1.116)	2.00	160.00	13.00 (3.604)
CD (0.05)	0.169			0.211
SEm±	0.055			0.068

Culture period - 3 weeks
* Average of three replications
** Values in parantheses represent transformed values
Medium - ½ MS supplemented with IAA and BAP 1.0 mg l^{-1}

Plate 19. Calli induced from mature leaf segments of black pepper cultivars and *Piper colubrinum*

From left to right - Kalluvally, Karimunda, Krishna (Panniyur-2), Cheriakanyakkadan, Panniyur-1, Balankotta and *P. colubrinum*



4.8.1.1.2 Calli induced from *in vitro* seedling explants

The percentage of callusing in explants of *in vitro* seedlings showed highly significant difference and vary between 55.55 to 92.22 (Table 31b). The highest percentage of callusing was recorded for Kalluvally (92.22) followed by Karimunda (85.92), both of them were found to be on par. The lowest callusing was exhibited by Panniyur-1 (55.55) which was found to be on par with Cheriakanyakkadan (66.66). Balankotta showed 75.92 per cent callusing which was on par with Cheriakanyakkadan and Karimunda.

The highest growth score of 2.73 was registered by Kalluvally followed by Balankotta (2.20). The lowest growth score was observed for Panniyur-1 (1.50).

The highest callus index (251.76) was observed for Kalluvally followed by Karimunda (171.84) and Balankotta (167.02). Panniyur-1 registered the lowest callus index of 83.33 followed by Cheriakanyakkadan (133.32).

The days taken for callusing (14.00 to 20.33) varied significantly among the cultivars. Early callusing was observed for Kalluvally (14.00) followed by Karimunda (15.33). Panniyur-1 showed late callusing (20.33).

4.8.1.1.3 Calli induced from *in vitro* seedling established cultures

When calli were induced from explants of *in vitro* seedling established cultures cent per cent callusing was observed for all the cultivars under study (Table 31c). The highest growth score of 3.00 and highest callus index of 300 were recorded for cultivars like Karimunda, Kalluvally and Balankotta.

Table 31b. Response of cultivars to callusing and callus growth in black pepper (Calli induced from *in vitro* seedling explants)

Cultivar	*Callusing (%)	*Growth score	Callus index	*Days for callus initiation
Panniyur-1	55.55 (0.842)**	1.50	83.33	20.33 (4.505)
Karimunda	85.92 (1.190)	2.00	171.84	15.33 (3.915)
Cheriakanyakkadan	66.66 (0.959)	2.00	133.32	16.00 (3.995)
Kalluvally	92.22 (1.295)	2.73	251.76	14.00 (3.737)
Balankotta	75.92 (1.059)	2.20	167.02	16.00 (3.996)
CD (0.05)	0.163			0.386
SEM±	0.052			0.125

Culture period - 3 weeks
* Average of three replications
** Values in parantheses represent transformed values
Medium - ½ MS supplemented with IAA and BAP 1.0 mg l^{-1}

Table 31c. Response of cultivars to callusing and callus growth in black pepper (Calli induced from *in vitro* seedling established cultures)

Cultivars	*Callusing (%)	*Growth score	Callus index	*Days for callus initiation
Panniyur-1	100	2.00	200.00	18.00 (0.424)**
Karimunda	100	3.00	300.00	12.00 (0.346)
Cheriakanyakkadan	100	2.80	280.00	13.33 (0.365)
Kalluvally	100	3.00	300.00	11.66 (0.341)
Balankotta	100	3.00	300.00	14.00 (0.374)
CD (0.05)				0.058
SEm±				0.018

Culture period - 3 weeks
* Average of three replications
** Values in parantheses represent transformed values
Medium - ½ MS supplemented with IAA and BAP 1.0 mg l^{-1}

The days taken for callusing showed significant difference and vary between 11.66 to 18.00. Early callusing was recorded in Kalluvally (11.66) followed by Karimunda (12.00), both of them were on par. Panniyur-1 took 18 days for callus initiation even in explants of *in vitro* established cultures.

4.8.1.1.4 Response of black pepper cultivars (irrespective of the source of explant) to callus induction and proliferation

Irrespective of the source of explant, based on callusing and callus growth the cultivars could be rated as follows - Kalluvally > Karimunda > Balankotta > Cheriakanyakkadan > Panniyur-1. With respect to days taken for callusing, Panniyur-1 showed late callusing (16.44) while all the other cultivars showed callusing between 12-14 days after inoculation (Table 31d, Fig.6 and 7).

4.8.1.1.5 Effect of source of explants on callusing and callus growth

Explants taken from *in vitro* established cultures showed cent per cent callusing and differ significantly from explants taken from *in vitro* seedlings and mature leaf segments (Table 31e). Irrespective of the cultivars, the percentage of callusing observed for explants from mature leaves and *in vitro* seedlings were on par.

With respect to the mean number of days taken for callusing, explants from mature leaf segments and *in vitro* established cultures were found to be on par taking 11.80 and 13.80 days for callusing respectively. The explants of *in vitro* seedling callused late and significantly differ from the other two taking 16.33 days for callusing.

Table 31d. Response of cultivars (irrespective of the source of explant) to callusing and callus growth in black pepper*

Cultivars	Callusing (%)	Growth score	Callus index	Days for callus initiation
Panniyur-1	80.49	1.72	141.98	16.44
Karimunda	92.96	2.46	230.72	13.33
Cheriakanyakkadan	81.84	2.28	191.67	13.78
Kalluvally	97.40	2.82	274.92	12.00
Balankotta	91.97	2.73	209.01	14.33

* Results reproduced from tables 31a, 31b and 31c

Fig. 6 Response of black pepper culltivars to callusing

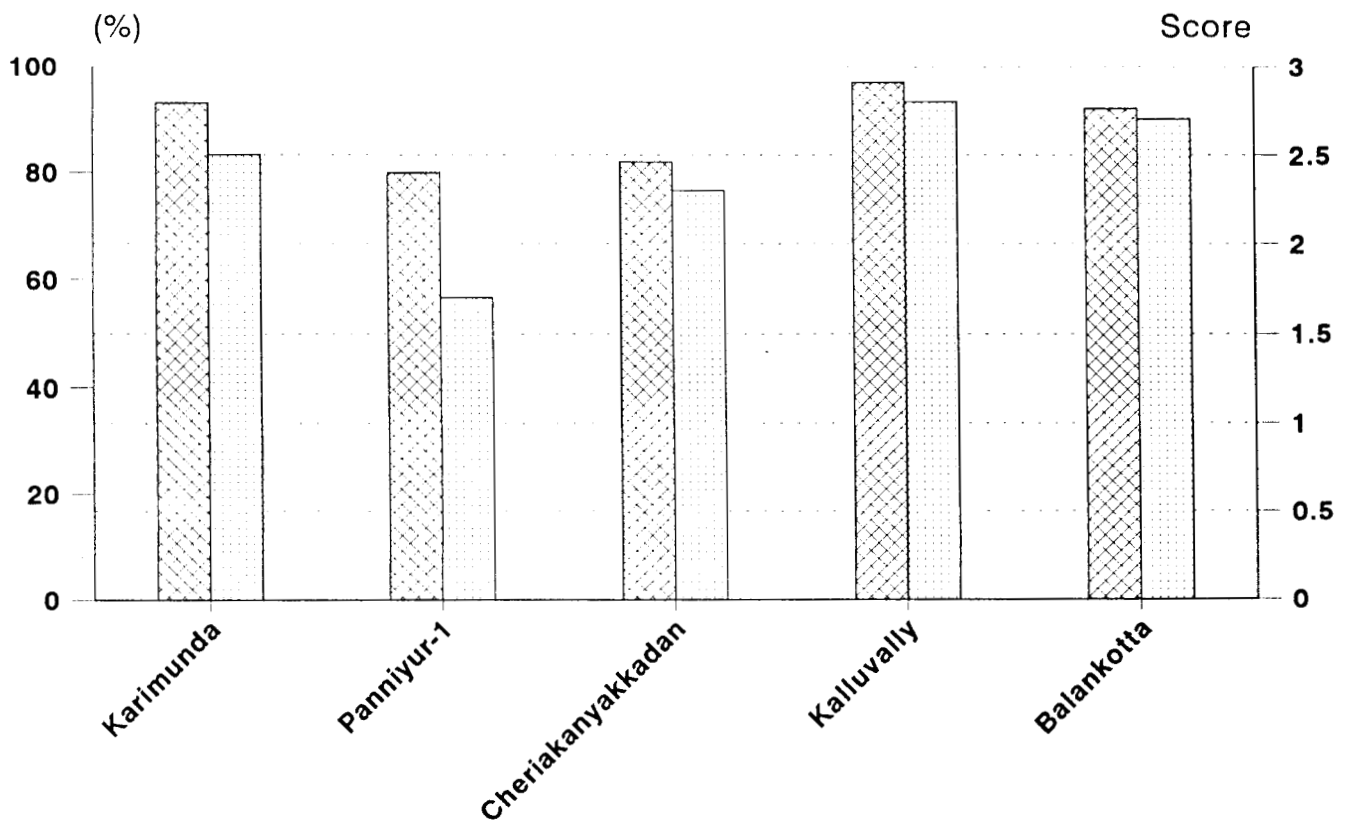
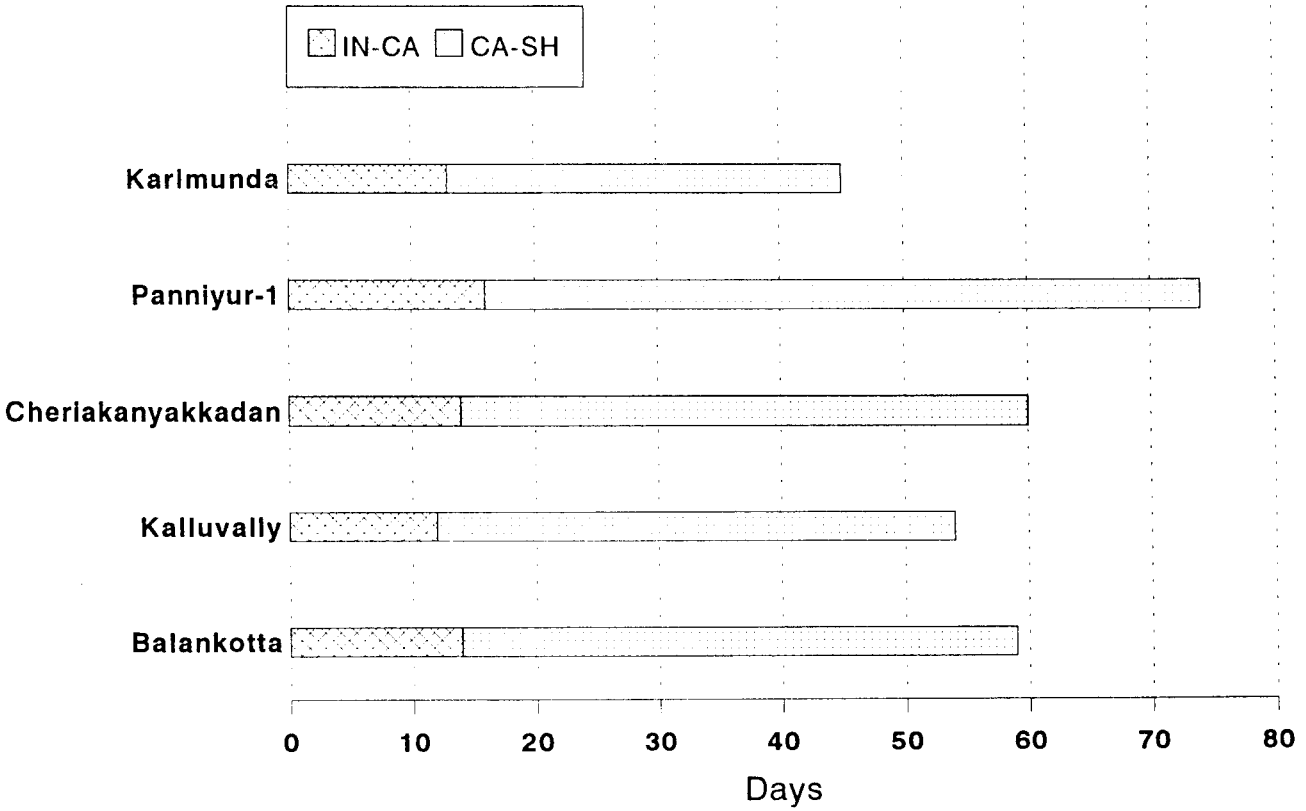


Fig. 7 Response of black pepper cultivars to callus/shoot initiation



IN-CA = Inoculation to callus initiation
CA-SH = Callus initiation to shoot regeneration

Table 31e. Effect of source of explants on callusing and callus growth in black pepper

Source of explants	Callusing (%)	Days for callus initiation	Callus index
Mature leaves	87.52 (1.215)*	11.80 (3.432)*	191.53
In vitro seedling	75.25 (1.066)	16.33 (4.033)	161.45
In vitro seedling established cultures	100.00 (1.455)	13.80 (3.703)	276.00
CD (0.05)	0.195	0.373	
SEm ±	0.063	0.121	

Culture period - 3 weeks
* Values in parentheses represent transformed values

The highest callus index irrespective of the cultivars was recorded by explants from *in vitro* established cultures (276) followed by mature leaf segment (191.53) and *in vitro* seedlings (161.45).

4.8.1.2 Response of black pepper cultivars and source of explants to shoot regeneration from calli

Callus cultures from three sources of explants were observed for the regeneration of shoots. The cultures showing regeneration of shoots and the number of days taken for the induction of shoots are presented in Table 32 and Plate 20.

None of the calli induced from mature leaf segments putforth shoots.

Although early regeneration of shoots were observed in cultures from *in vitro* established seedling explants compared to *in vitro* seedlings, χ^2 analysis showed that the differences were not statistically significant. Similarly slightly higher percentage of regeneration was recorded in cultures from *in vitro* established explants, the differences were not statistically significant.

The cultivars differed significantly in the number of days taken for shoot induction and number of cultures showing regeneration of shoots. Karimunda showed highest regeneration potential giving shoot induction in 68.51 per cent of the cultures followed by Kalluvally in 63.33 per cent of the cultures, both of them were found to be on par. Balankotta and Cheriakanyakkadan were also found to be on par giving 57.78 and 54.63 per cent regeneration of shoots respectively. Panniyur-1 registered the lowest regeneration of shoots giving 33.33 per cent, which differed significantly from all other cultivars (Fig.8).

Table 32. Response of cultivars and source of explants to shoot regeneration from calli

Varieties	Days for shoot induction			Cultures showing regeneration of shoots in 2nd subculture (%)		
	In vitro seedling	*In vitro* seedling established cultures	Variety mean	*In vitro* seedling	*In vitro* seedling established cultures	Variety mean
Panniyur-1*	60.00	56.66	58.33 (1.635)**	33.33	33.33	33.33 (0.612)*
Karimunda	33.33	31.33	32.33 (5.685)	66.66	70.36	68.51 (0.976)
Kalluvally	43.00	41.66	42.33 (6.504)	60.00	66.66	63.33 (0.923)
Balankotta	45.66	43.33	44.50 (6.670)	55.55	60.00	57.78 (0.865)
Cheriyakanya-kkadan	47.33	44.33	45.83 (6.768)	53.70	55.55	54.63 (0.832)
CD (0.05)			0.209			0.106
SEm±			0.072			0.037

Culture period - 3 weeks

* The percentage of regeneration in 3rd subculture

** Values in parentheses represent transformed values

χ^2 value for comparison of the source of explants with the days taken for shoot initiation - 0.923^{NS}

χ^2 value for comparison of the source of explants with the % of cultures showing regeneration - 0.0133^{NS}

Fig. 8 Response of black pepper cultivars to shoot regeneration from calli

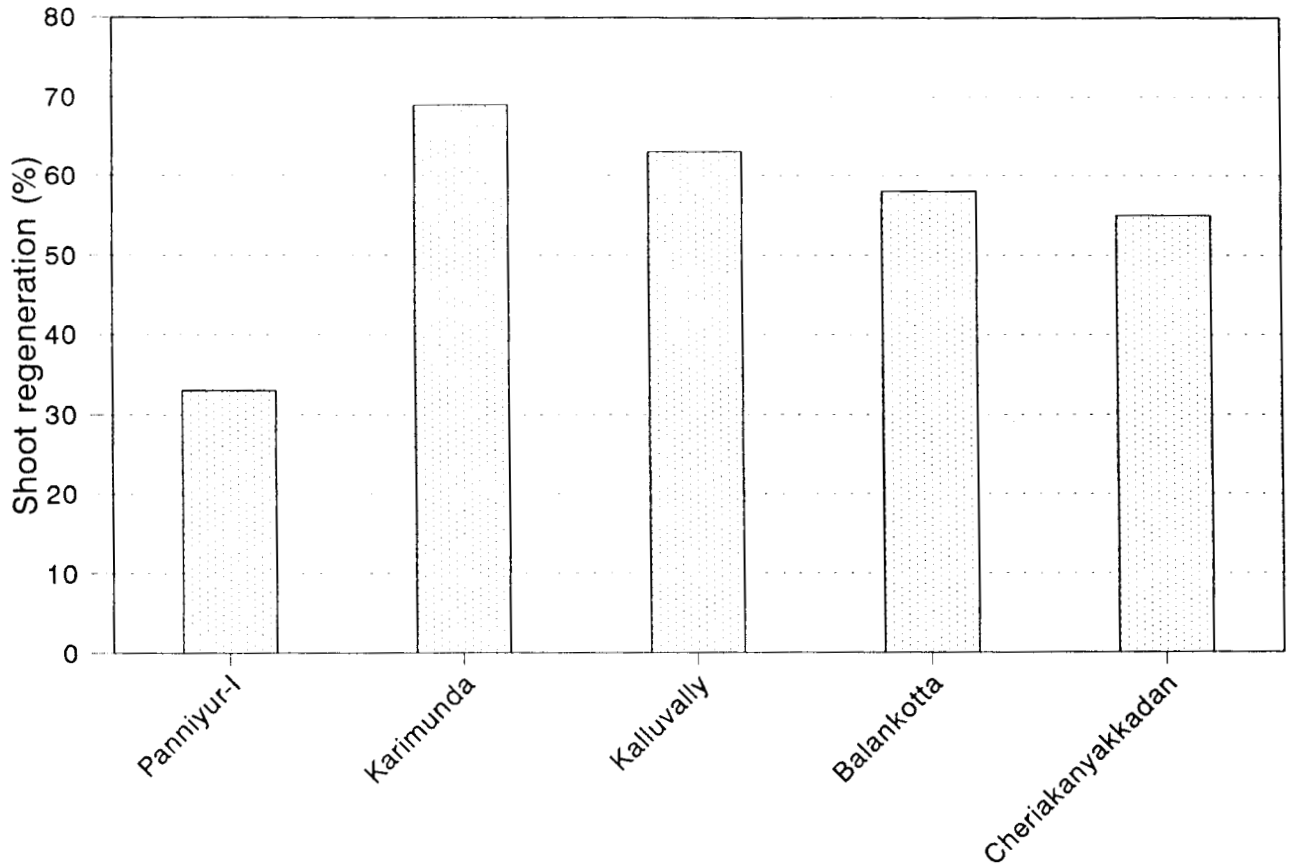


Plate 20. Regeneration of shoots from black pepper calli

With respect to the days taken for induction of shoots after callusing, early induction was observed in the cultivar Karimunda (32.33 days) while Panniyur-1 took 58.33 days for the induction of shoots. Other cultivars studied viz. Kalluvally, Balankotta and Cheriakanyakkadan took more or less the same period (42.33-45.83 days) for induction of shoots (Fig.7).

4.8.1.3 Effect of containers on shoot proliferation

The influence of different containers in the proliferation of shoots revealed that big culture tubes of dimension 380 x 200 mm were the best giving 16.5 proliferated shoots (Plate 21) followed by 100 ml Erlenmeyer flask (13.5). However, 250 ml jars and 250 ml Erlenmeyer flasks were found inferior with respect to the proliferation of shoots in the 4th subculture (Table 33).

4.8.1.4 Response of black pepper cultivars to shoot proliferation

The cultivars differed significantly in their rate of proliferation of shoots (Table 34). The highest proliferation of shoots was recorded by Kalluvally (18.16) followed by Karimunda (16.58), Cheriakanyakkadan (15.66) and Balankotta (11.33). Panniyur-1 was inferior to all other cultivars registering lowest proliferation of 3.91. However, Karimunda and Cheriakanyakkadan were on par with respect to proliferation of shoots.

4.8.1.5 Response of black pepper cultivars to the recovery of rootable shoots

Comparative performance of cultivars to the recovery of rootable shoots in 4th, 5th and 6th subcultures is presented in Table 35.

Plate 21. Highly proliferating culture in big culture tube

Table 33. Effect of containers on shoot proliferation in black pepper (cv. Karimunda)

Containers	* No. of shoots further proliferated in 3rd subculture
1. Culture tube - small (15 x 2.5 cm)	7.16 (2.671)
2. Erlenmeyer Flask - 100 ml	13.50 (3.664)
3. Erlenmeyer Flask - 250 ml	8.16 (2.852)
4. Culture tube - big (380 x 200 mm)	16.50 (4.064)
5. Jars - 250 ml	9.33 (3.043)
CD (0.05)	0.2031
SEm±	0.071

Culture period - 3 weeks
* Average of 12 observations
Medium - ½ MS supplemented with IAA and BAP 1.0 mg l^{-1}

Table 34. Response of cultivars to shoot proliferation in black pepper

Cultivars	*No. of shoots further proliferated in 3rd subculture	Highest number noted in 3rd subculture
1. Karimunda	16.58 (4.066)**	25
2. Kalluvally	18.16 (4.255)	38
3. Balankotta	11.33 (3.357)	14
4. Cheriakanyakkadan	15.66 (3.946)	28
5. Panniyur-1***	3.91 (1.968)	6
CD (0.05)	0.216	
SEm±	0.076	

Culture period - 3 weeks
Container - Big culture tubes (380 x 200 mm)
* Average of 12 observations
** Values in parantheses represent transformed values
*** Number of shoots proliferated in the 4th subculture

Table 35. Response of cultivars to the recovery of rootable shoots in black pepper

Cultivars	* No. of rootable shoots			Highest number noted in 6th subculture
	4th subculture	5th subculture	6th subculture	
Karimunda	1.25 (1.47)**	2.91 (1.939)	5.00 (2.439)	8.0
Kalluvally	2.50 (1.852)	8.00 (2.991)	12.00 (3.600)	15.0
Balankotta	1.16 (1.443)	7.08 (2.833)	9.33 (3.211)	12.0
Cheriakanyakkadan	1.916 (1.689)	7.16 (2.852)	10.25 (3.349)	13.0
Panniyur-1	0	1.00 (1.390)	2.08 (1.734)	3.0
CD (0.05)	0.251	0.230	0.184	
SEm±	0.084	0.081	0.065	

Culture period - 3 weeks
* Average of 12 observations
** Values in paranetheses represent transformed values
Medium - ½ MS supplemented with IAA 0.1 and BAP 0.2 mg l^{-1}

Kalluvally registered the highest recovery of rootable shoots in all sub-cultures followed by Cheriakanyakkadan. The mean number of rootable shoots in 6th subculture for Kalluvally, Cheriakanyakkadan and Balankotta were 12, 10.25 and 9.33 respectively. All the cultivars except Balankotta and Cheriakanyakkadan differed significantly with respect to recovery of rootable shoots (Fig.9 and Plates 22a to 22e). The lowest recovery of rootable shoots (2.05) was recorded by Panniyur-1 followed by Karimunda (5.00).

4.8.1.6 Response of black pepper cultivars to rooting and root growth

The five cultivars studied recorded cent per cent rooting (Table 36 and Plate 23).

All the cultivars except Panniyur-1 took 12.75 to 13.83 days for the root initials to appear while Panniyur-1 took 15 days. Kalluvally, Balankotta, Cheria-kanyakkadan and Karimunda differed significantly from Panniyur-1 while four of them were on par.

The cultivars studied showed highly significant variation in root number (Fig.10). The highest root number was recorded by Cheriakanyakkadan (17.66) closely followed by Kalluvally (15.83), both of them were on par. Balankotta registered a mean root number of 13.66 which was found to be on par with Karimunda (12.25). The lowest number of roots (8.75) was recorded by Panniyur-1.

Panniyur-1 differed significantly from all the other cultivars with respect to root length, all the other cultivars being on par. The highest mean root length of 3.04 cm was exhibited by Cheriakanyakkadan followed by Balankotta (2.79 cm),

Fig. 9 Response of black pepper cultivars to the recovery of rootable shoots

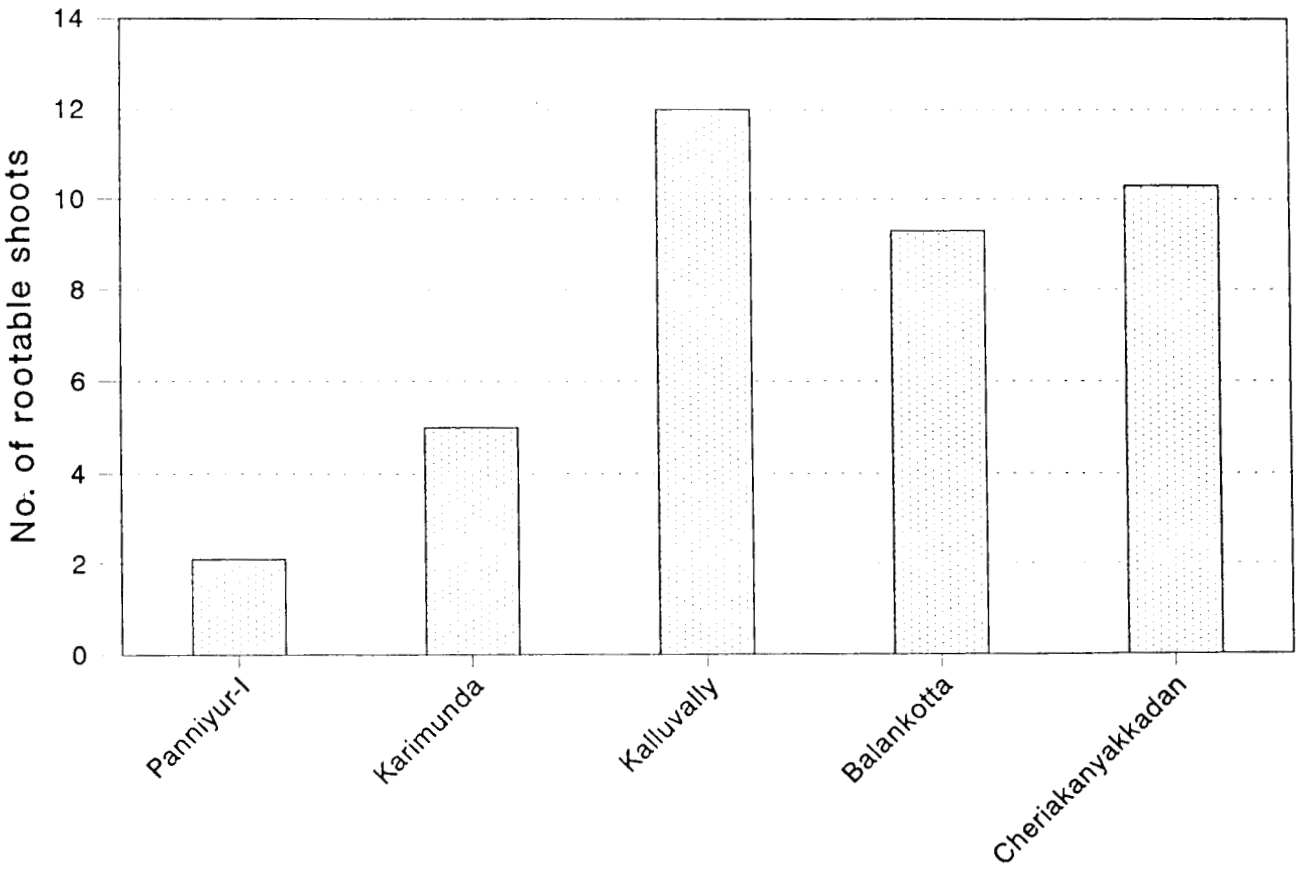
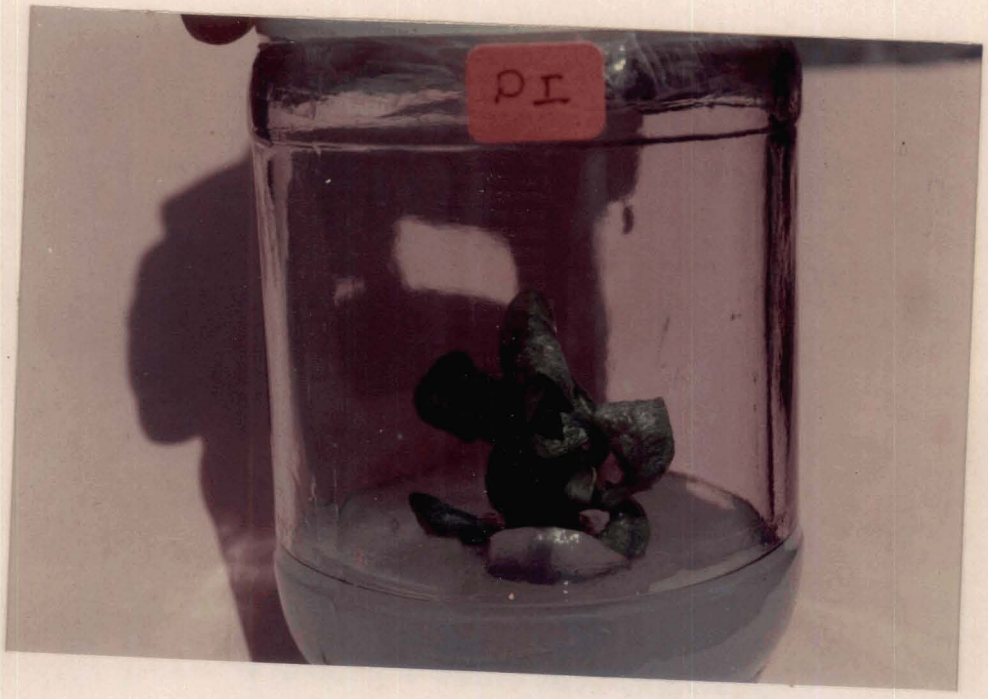


Plate 22(a-e). Recovery of rootable shoots in black pepper cultivars

a. Panniyur-1


P I

22 b. Kalluvally

22 c. Karimunda

22 d. Cheriakanyakkadan

22 e. Balankotta

Table 36. Response of cultivars to rooting and root growth in black pepper

Cultivars	Rooting (%)	Days * for root initials to appear	No. of roots*	Length of* roots (cm)	Thickness* of roots (mm)
1. Kalluvally	100	12.75 (3.707)**	15.83 (3.947)	2.75	1.02
2. Cheriakanyakkadan	100	13.50 (3.806)	17.66 (4.007)	3.04	1.27
3. Balankotta	100	13.83 (3.850)	13.66 (3.660)	2.79	1.08
4. Karimunda	100	13.16 (3.762)	12.25 (3.468)	2.64	1.12
5. Panniyur-1	100	15.00 (3.998)	8.75 (2.949)	2.19	0.90
CD (0.05)		0.086	0.376	0.543	0.151
SEm±		0.030	0.132	0.191	0.053

Culture period - 1 month
* Average of 12 observations
** Values in parantheses represent transformed values
Medium - ½ MS supplemented with sucrose 2 per cent and IBA 1.0 mg l^{-1}

Plate 23. Rooted plantlets of black pepper cultivars

BORAX
TEST TUBE
BORAX
TEST TUBE
20
25

Kalluvally (2.75 cm) and Karimunda (2.64 cm). Panniyur-1 registered the lowest root length of 2.19 cm.

The mean root thickness in different cultivars varied significantly and ranged from 0.9 to 1.27 mm in different cultivars (Fig.10). The highest root thickness was exhibited by Cheriakanyakkadan which differed significantly from all other cultivars. Karimunda registered a root thickness of 1.12 mm closely followed by Balankotta (1.08 mm) both of them were on par. Kalluvally registered a root thickness of 1.02 mm which was found to be on par with Panniyur-1 (0.90 mm).

4.9 Planting out, hardening and final survival

4.9.1 Effect of season and weather parameters on final survival of plantlets

The data relating to the total number of plantlets planted out, number wilted and final survival is presented in Appendix-II and the monthly weather data at the experimental site from 1992-1994 is presented in Appendix-III.

The monthwise planting out of the regenerants from January 1994 to September 1994 is presented in Table 37.

The highest survival was recorded in July (61.06%) followed by September (53.29%). The χ^2 values showed highly significant variation in the mortality and final survival of plantlets between the different months in which planting out was done. Mortality of plants during hardening is shown in plates 24a to 24c.

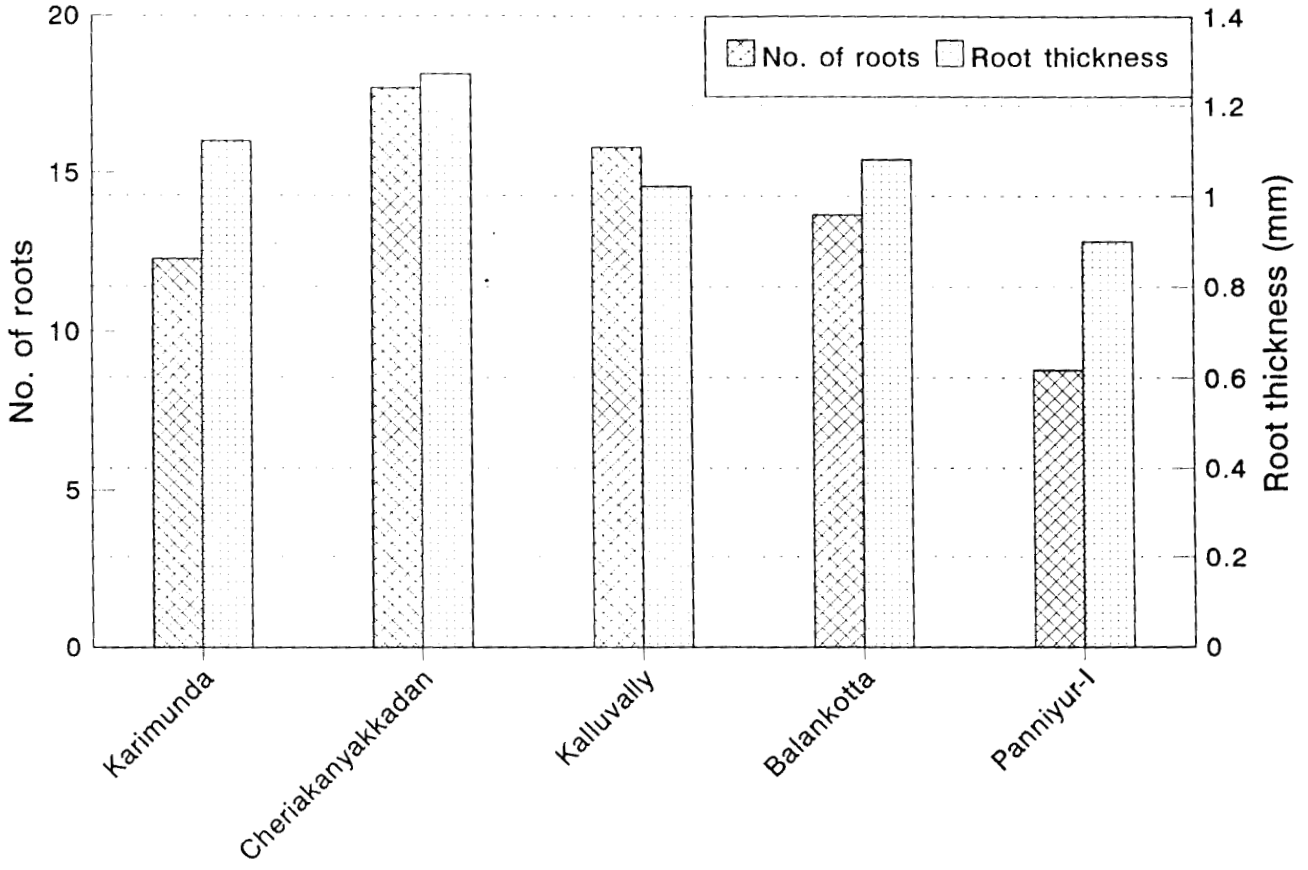
The correlation coefficients worked out with weather parameters and mortality and final survival revealed that the mortality of plants during and after

Table 37. Effect of season on final survival of black pepper plantlets

Month	Mortality of plants during hardening (%)	Mortality of plants after hardening (%)	Final survival (%)
1. January 1994	68.18	9.09	22.73
2. February 1994	58.33	2.78	38.88
3. March 1994	50.00	11.11	38.89
4. April 1994	42.86	7.14	50.00
5. May 1994	60.15	4.34	35.51
6. June 1994	44.66	7.77	47.57
7. July 1994	27.14	11.80	61.06
8. August 1994	41.77	18.99	39.24
9. September 1994	32.87	13.84	53.29
χ^2 values for comparison of effect of months from January 1994 to September 1994	139.457**	42.030**	156.751**

Plate 24(a-c). Mortality of plants during hardening

Fig. 10 Response of black pepper cultivars to root growth



hardening showed a positive correlation with mean relative humidity percentage (Appendix-IV). The mortality of plants after hardening was also positively correlated with sunshine hours. The final survival of the plantlets was positively correlated with rainfall, number of rainy days and relative humidity and negatively to sunshine hours. Hardened plantlets are shown in plate 25.

4.9.2 Effect of callus screening on final survival of plantlets

Data relating to the total number of plantlets planted out from screened and unscreened calli, mortality of plants during and after hardening and final survival is presented in Appendix-V.

The effect of screening the calli on the mortality and final survival of plantlets is presented in Table 38 and Fig.11. The plantlets regenerated from screened cultures showed high mortality and low final survival. However, comparison by 't' test showed that the differences were not statistically significant.

4.9.3 Influence of cultivars on final survival of plantlets

The χ^2 analysis showed that varieties differed significantly in the mortality and final survival of plantlets (Table 39). The highest mortality during hardening was observed in Balankotta (52.17%) followed by Kalluvally (41.62%), Karimunda (38.98%), Panniyur-1 (34.62%) and the lowest by Cheriakanyakkadan (30.34%).

Mortality of plants was observed after hardening also. Out of the plantlets survived after hardening, the lowest mortality was observed for Cheria-kanyakkadan (9.90%) followed by Karimunda (11.11%). The other cultivars namely

Table 38. Effect of callus screening on the survival of regenerated black pepper plantlets

Cultivars	Mortality during hardening (%)		Mortality after hardening (%)		Final survival (%)	
	Screened	Unscreened	Screened	Unscreened	Screened	Unscreened
Karimunda	66.66	38.98	14.28	11.11	28.57	54.24
Balankotta	48.78	52.17	33.33	27.27	34.14	34.78
Cheriakani-kkadan	33.33	30.34	11.90	9.90	58.73	62.75
Kalluvally	42.86	41.62	21.43	20.37	44.90	46.49
Culture No.I	42.29	30.48	22.13	17.54	44.93	57.32
t value	1.1831		0.6940		1.2488	
Probability	0.2707		0.5074		0.2471	

χ^2 values for comparison of cultivars vs. survival of screened calliclones - 21.692**

χ^2 values for comparison of cultivars vs. survival of unscreened calliclones - 19.371**

Fig. 11 Effect of callus screening on final survival of black pepper plantlets

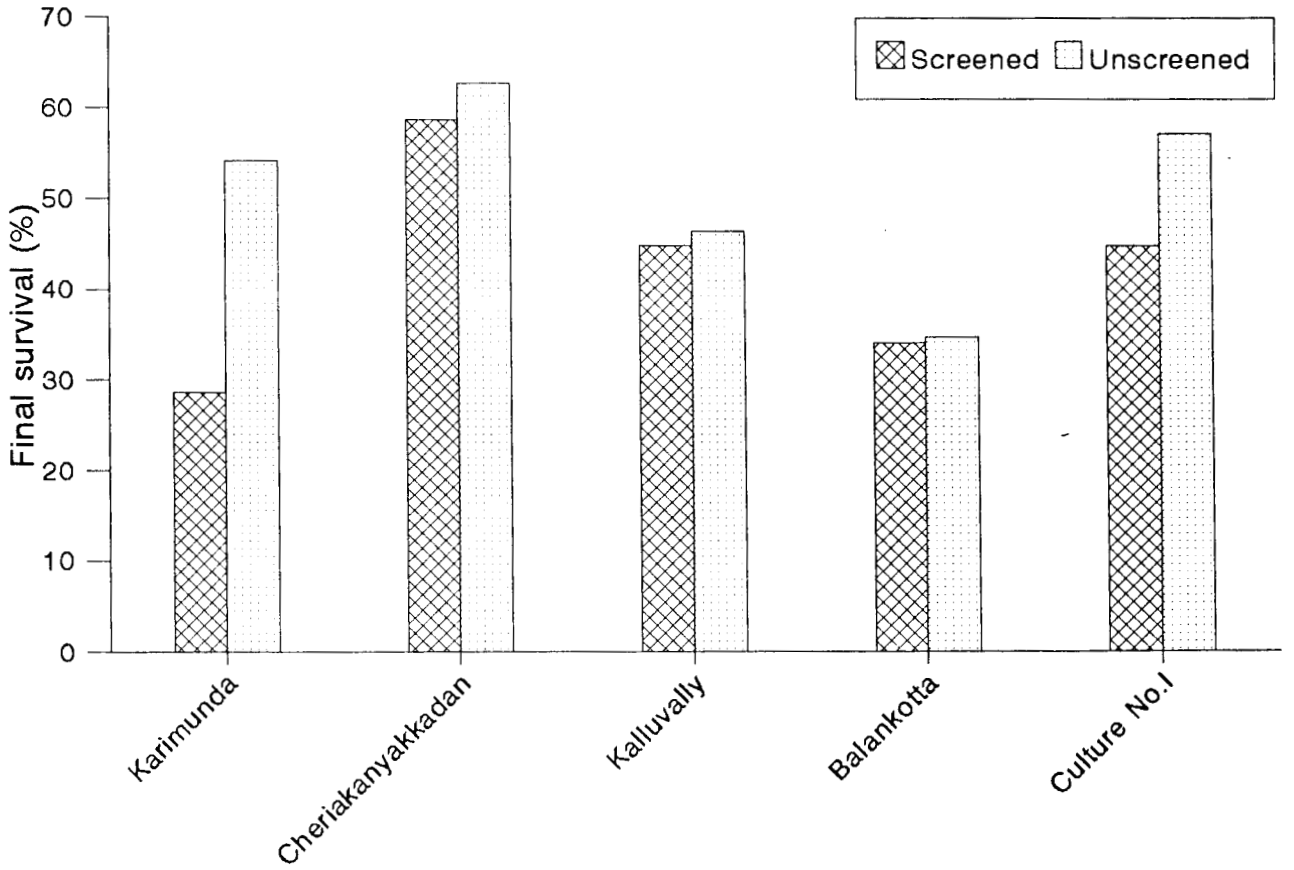


Table 39. Response of cultivars to final survival of black pepper plantlets

Cultivars	Mortality during hardening (%)	Mortality after hardening (%)	Final survival* (%)
Karimunda	38.98	11.11	54.24
Panniyur-1	34.62	29.41	46.15
Balankotta	52.17	27.27	34.78
Cheriakanyakkadan	30.34	9.90	62.76
Kalluvally	41.62	20.37	46.49
x^2 values for comparison of cultivars	11.846**	22.989**	17.593**

* Final survival worked out, from the plantlets survived after hardening

Panniyur-1, Balankotta and Kalluvally showed 20.37 to 29.41 per cent mortality after hardening.

The highest final survival was recorded for Cheriakanyakkadan (62.76%) followed by Karimunda (54.24%), Kalluvally (46.49%), Panniyur-1 (46.15%) and Balankotta (34.78%). Cheriakanyakkadan showed the lowest mortality and highest final survival followed by Karimunda (Plate 26).

4.10 Screening the calliclones for tolerance/resistance to *Phytophthora capsici*

4.10.1 Natural screening for resistance to *P. capsici* (keeping in infected field)

All the 312 plants kept for natural screening took infection and wilted completely, showing that none of the calliclones were completely resistant to the disease (Plates 27 and 28a to 28c).

4.10.1.1 Effect of callus screening on the development of symptoms

Eventhough all the 312 plants kept for natural screening took infection and wilting, the unscreened plants registered slightly lower rate of wilting when compared to the screened ones, but the difference was not statistically significant (Table 40).

Within a period of six days 48.39 per cent of plants in the screened group wilted while only 42.86 per cent of the plants in the unscreened group showed the symptom. Similarly on the 9th day 69.35 per cent of the plants in the screened group wilted while only 67.46 per cent of the plants in the unscreened group showed the symptom.

Plate 25. Hardened plantlets of black pepper cultivars

Plate 26. Established calliclones of black pepper

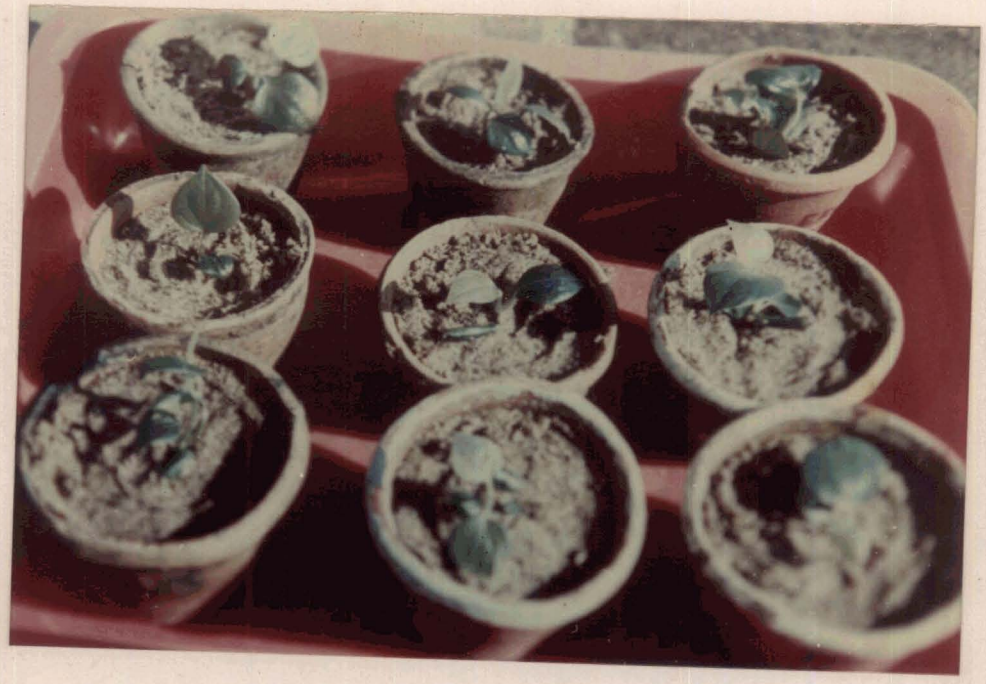


Plate 27. Calliclones kept in the infected field for natural screening

Plate 28. Symptoms produced in natural screening

28a. Foliar infection

28b. Tip infection

28c. Collar infection

Table 40. Effect of callus screening on development of symptoms in regenerated calliclones (natural screening)

Treatment	No. of plants kept	Plants wilted on each day (%)										
		4th	5th	6th	7th	8th	9th	10th	11th	12th	13th	14th
Screened	186	3.23	24.73	20.43	9.14	6.99	4.84	10.22	9.14	2.15	5.38	3.76
Unscreened	126	3.97	22.22	16.67	7.94	10.32	6.35	10.32	7.14	2.38	7.14	5.55

t value for comparison of screened Vs unscreened = 0.1286
Probability = 0.8989

4.10.1.2 Effect of age of the calliclones on symptom development

There was no significant difference observed between the different age groups in the number of plants wilted due to infection on each day (Table 41). All the plants wilted within a period of 10 days in plants of age groups, three to six months and two to three months. On the other hand plants of the age group, one to two months took 14 days for complete infection and wilting. This was because the rainfall and relative humidity during the period was low and there was total absence of rain for three days during the period of study.

4.10.1.3 Screening the calliclones by inducing electrolyte leakage with concentrated culture filtrate

Since none of the regenerated calliclones showed complete resistance to the disease, the tolerance level of the clones was tested by electrolyte leakage method.

4.10.1.3.1 Effect of callus screening on electrolyte leakage from regenerated calliclones

The clones derived from screened and unscreened calli were subjected to electrolyte leakage studies. Comparison of electrolyte leakage values from the two sources showed that there was no significant difference in cultivars like Karimunda, Kalluvally and Balankotta (Table 42). But in the cultivar Cheriakanyakkadan leakages from the two sources showed highly significant variation recording a higher leakage of 63.5 μmhos in clones derived from screened calli and 51.42 μmhos in clones derived from unscreened calli.

Table 41. Effect of age of the regenerated calliclones on development of symptoms (natural screening)

Age groups	No. of plants kept	Plants wilted on each day (%)											Mean
		4th	5th	6th	7th	8th	9th	10th	11th	12th	13th	14th	
3-6 months	43	9.30	37.20	20.93	13.95	6.98	4.65	6.98	-	-	-	-	NS (0.368)
2-3 months	91	5.49	31.87	36.26	8.79	8.79	3.30	5.49	-	-	-	-	NS (0.358)
1-2 months	178	1.12	16.29	9.55	7.30	8.43	6.74	13.48	14.61	3.93	10.67	7.87	NS (0.296)

Table 42. Effect of callus screening on elctrolyte leakage from regenerated black pepper calliclones

Cultivars	Mean electrolyte leakage μmhos		t value	Probability
	Screened	Unscreened		
Karimunda	82.42	73.85	1.5810	0.425
Cheriakanyakkadan	63.50	51.42	2.7047	0.0221
Kalluvally	72.33	79.50	0.7630	0.410
Balankotta	63.42	61.60	0.8244	0.380

4.10.1.4 Screening the regenerated calliclones by artificial inoculation of culture disc of *P. capsici*

The tolerance level of the calliclones to the disease was assessed based on the intensity of lesion development. The variation in lesion development is shown in Plates 29a to 29d.

4.10.1.4.1 Effect of callus screening on intensity of lesion development in regenerated calliclones

The intensity of lesion development in regenerants from screened and unscreened calli is presented in Table 43 and Fig.12.

The screened and unscreened regenerated calliclones were compared for lesion development, irrespective of the cultivars. Majority of the plants regenerated from screened calli were coming in the 3rd class giving an average lesion diameter of 1.1 to 1.5 cm while majority of the plants from unscreened calli were coming in the 2nd class having less average lesion diameter (0.5-1.0 cm). Likewise 4.9 per cent of the plants in the screened group represented the 5th class having the highest lesion diameter (> 2.0 cm) while only 2.11 per cent of plants of the unscreened group represented the 5th class.

The overall lesion diameter score when compared, it was found that the regenerants from the screened group registered a score of 296.51 which was 11.10 per cent higher than that recorded in regenerants from unscreened calli.

4.10.1.4.2 Response of black pepper cultivars to intensity of lesion development in regenerated calliclones

The influence of cultivars on intensity of lesion development was evaluated in two cultivars namely Kalluvally and Cheriakanyakkadan where there

Plate 29a to 29d. Variation in lesion development in regenerated callicones. Screening done by artificial inoculation of culture disc of *Phytophthora capsici*

29a

29b


FP-12
FP-26

Table 43. Effect of callus screening on intensity of lesion development in regenerated black pepper calliclones

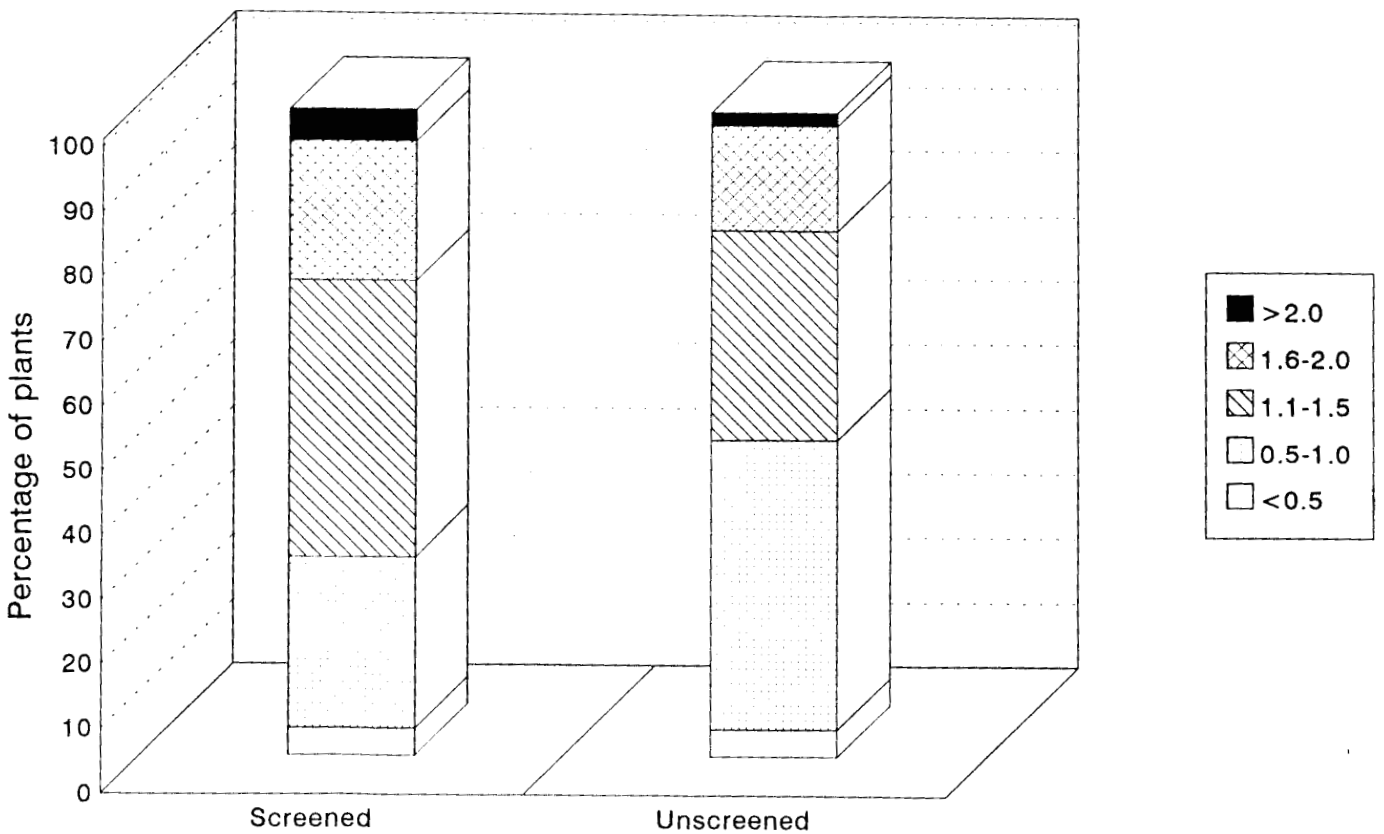
Class based on lesion diameter (cm)	Screened*		Unscreened**	
	Percentage of plants	LDS***	Percentage of plants	LDS***
1. < 0.5	4.2	4.2	4.23	4.23
2. 0.5 - 1.0	26.57	53.14	45.07	90.14
3. 1.1 - 1.5	42.65	127.95	32.39	97.17
4. 1.6 - 2.0	21.68	86.72	16.20	64.80
5. > 2.0	4.90	24.50	2.11	10.55
Total LDS		296.51		266.89

* No. of plants observed for intensity of lesion development - 143
** No. of plants observed for intensity of lesion development - 142

*** Lesion diameter score (LDS) calculated by multiplying the % of plants in each group with the score. The score was worked out based on a scale 1 to 5

where 1 = < 0.5 cm lesion diameter, 2 = 0.5 - 1.0 cm lesion diameter
3 = 1.1 - 1.5 cm lesion diameter, 4 = 1.6 - 2.0 cm lesion diameter
5 = > 2.0 cm lesion diameter

Fig. 12 Effect of callus screening on lesion development in calli clones



were more number of regenerants and the results are presented in Table 44 and Fig.13.

The percentage of plants coming under each class and the LDS were compared between the two cultivars. The cultivar Cheriakanyakkadan was having higher percentage (5.63) of plants showing lesion diameter less than 0.5 cm as compared to Kalluvally (3.64%). Similarly majority of the plants of Cheriakanyakkadan came in the second class having average lesion diameter ranging from 0.5 to 1.0 cm, while most of the plants of Kalluvally came in the second and third classes having higher average lesion diameter, 0.5 to 1.0 cm and 1.1 to 1.5 cm.

The overall lesion diameter score recorded for Kalluvally was 289.09 which was 14.68 per cent higher than that recorded for Cheriakanyakkadan (252.08).

4.10.1.4.3 Effect of callus screening on intensity of lesion development in regenerated calliclones of cultivars Kalluvally and Cheriakanyakkadan

The lesion diameter developed in screened and unscreened calliclones of Kalluvally was compared and the results are presented in Table 45. Screened clones by double layer culture (Method 3) in Kalluvally exhibited 4.40 per cent higher lesion development score compared to the unscreened ones. However, clones originated after screening by method 1, registered less LDS compared to the unscreened ones. The percentage of plants coming in each class when examined, the screened clones were giving more susceptible plants having higher number of plants in the 3rd, 4th and 5th classes compared to the unscreened ones where 1st, 2nd and 3rd classes represented higher number of plants.

Table 44. Response of black pepper cultivars to intensity of lesion development in regenerated calliclones

Class based on lesion diameter in cm	Cultivars			
	Kalluvally*		Cheriakanyakkadan*	
	Percentage of plants	LDS**	Percentage of plants	LDS**
1. < 0.5	3.64	3.64	5.63	5.63
2. 0.5 - 1.0	34.54	69.08	52.11	104.22
3. 1.1 - 1.5	32.73	98.19	29.58	88.74
4. 1.6 - 2.0	27.27	109.08	9.86	39.44
5. > 2.0	1.82	9.10	2.81	14.05
Total LDS		289.09		252.08

* No. of calliclones screened - Cheriakanyakkadan - 71
- Kalluvally - 55

** Lesion diameter score (LDS) calculated by multiplying the percentage of plants in each group with the score. Score was worked out based on a scale 1 to 5 where

1 = < 0.5 cm lesion diameter, 2 = 0.5 - 1.0 cm lesion diameter
3 = 1.1 - 1.5 cm lesion diameter, 4 = 1.6 - 2.0 cm lesion diameter
5 = > 2.0 cm lesion diameter

Fig. 13 Response of black pepper cultivars to lesion development

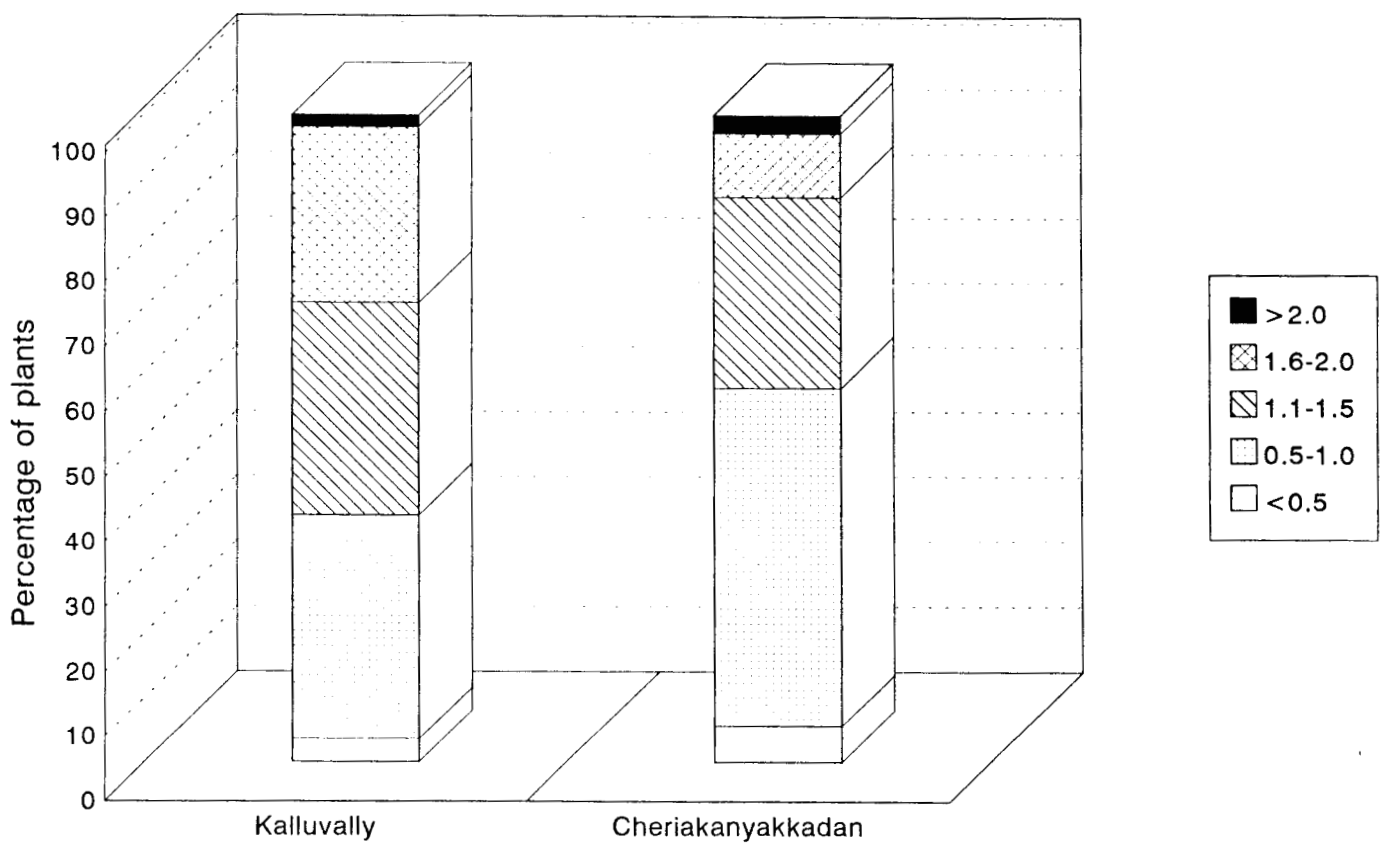


Table 45. Effect of callus screening on intensity of lesion development in regenerated calliclones of Kalluvally and Cheriakanyakkadan

Class based on lesion diameter in cm	Kalluvally						Cheriakanyakkadan			
	Screened (Method 1)		Screened (Method 3)		Unscreened		Screened		Unscreened	
	Percentage of plants	LDS*	Percentage of plants	LDS*	Percentage of plants	LDS*	Percentage of plants	LDS*	Percentage of plants	LDS*
< 0.5	0	0	3.60	3.60	3.63	3.63	20.00	20.00	5.63	5.63
0.5 - 1.0	46.67	93.34	26.13	52.26	34.54	69.08	20.00	40.00	52.11	104.22
1.1 - 1.5	46.67	140.01	40.54	121.62	32.73	98.19	50.00	150.00	29.58	88.74
1.6 - 2.0	6.67	26.68	24.32	97.28	27.27	109.08	10.00	40.00	9.86	39.44
> 2.0	0	0	5.41	27.05	1.82	9.10	0	0	2.81	14.05
Total LDS		260.03		301.81		289.08		250.00		252.08

* Lesion diameter score (LDS) calculated by multiplying the percentage of plants in each group with the score. Score was worked out based on a scale 1 to 5 where

1 = < 0.5 cm lesion diameter, 2 = 0.5-1.0 cm lesion diameter
3 = 1.1-1.5 cm lesion diameter, 4 = 1.6-2.0 cm lesion diameter
5 = > 2.0 cm lesion diameter

In the case of Cheriakanyakkadan the LDS registered for clones from the screened and unscreened calli were almost the same. The percentage of plants coming in each class showed that the screened calli derived clones are more susceptible compared to the unscreened ones having higher number of plants in classes with higher lesion diameter.

4.10.1.4.4 Effect of different methods of callus screening on intensity of lesion development in regenerated Kalluvally calliclones

The three methods of screening tried were compared based on the LDS recorded in Kalluvally calliclones (Table 46). The method 1 in which calli were grown in CCF incorporated medium recorded the lowest LDS (260.03) followed by calliclones screened by method 3 and method 2. Regenerants from calli screened by method 3 registered 16.06 per cent higher LDS than method 1. The regenerants from method 2 registered 46.14 per cent higher LDS than method 1.

The percentage of plants coming under each class when examined, it was found that regenerants from calli screened by method 1 and 2 were giving more susceptible plants having higher LDS while regenerants from calli screened by method 3 were giving tolerant plants with less LDS.

4.11 Comparison of somaclonal variation in black pepper cultivars

4.11.1 Somaclonal variation in electrolyte leakage

Electrolyte leakage was induced from leaves of the somaclones of four cultivars viz. Kalluvally, Balankotta, Cheriakanyakkadan and Karimunda using CCF. The leakage values from the somaclones are presented in Table 47. The χ^2 analysis showed that there was no significant somaclonal variation in electrolyte

Table 46. Effect of method of callus screening on intensity of lesion development in regenerated Kalluvally calliclones

Class based on lesion diameter in cm	Method 1		Method 2		Method 3	
	Percentage of plants	LDS*	Percentage of plants	LDS*	Percentage of plants	LDS*
1. < 0.5 cm	0	0	0	0	3.60	3.60
2. 0.5 - 1.0	46.67	93.34	0	0	26.13	52.26
3. 1.0 - 1.5	46.67	140.01	40.00	120.00	40.54	121.62
4. 1.6 - 2.0	6.67	26.68	40.00	160.00	24.32	97.28
5. > 2.0	0	0	20.00	100.00	5.41	27.05
Total LDS		260.03		380.00		301.81

* Lesion diameter score (LDS) calculated by multiplying the percentage of plants in each group with the score. Score was worked out based on a scale 1 to 5 where

1 = < 0.5 cm lesion diameter, 2 = 0.5-1.0 cm lesion diameter
2 = 1.1-1.5 cm lesion diameter, 4 = 1.6-2.0 cm lesion diameter
5 = > 2.0 cm lesion diameter

Table 47. Somaclonal variation in electrolyte leakage from regenerated calliclones of black pepper cultivars

Soma-clones	Electrolyte leakage over control in μmhos			
	Kalluvally	Cheriakanyakkadan	Balankotta	Karimunda
S_1	83	52	63	82
S_2	118	60	56	75
S_3	98	59	55	71
S_4	59	50	74	75
S_5	97	41	60	68
S_6	72	47	75	73
S_7	84	51	58	73
S_8	65	59	62	69
S_9	59	54	54	70
S_{10}	60	50	61	83
χ^2 values for comparison	45.919**	6.117	7.823	3.179

leakage in cultivars like Cheriakanyakkadan, Balankotta and Karimunda (Fig.14). But highly significant variation was observed in the cultivar Kalluvally giving a χ^2 value of 45.92.

4.11.2 Somaclonal variation in lesion development

Somaclones of two cultivars namely Kalluvally and Cheriakanyakkadan were observed for the somaclonal variation in lesion development (Table 48). In Cheriakanyakkadan 64.71 per cent of plants came in one class (0.5-1.0 cm) which indicated that the cultivar was giving more uniform plants with respect to the character tested. In Kalluvally majority of the plants were distributed in three classes (0.5-1.0, 1.1-1.5 and 1.6-2.0 cm) showing the variability in the character tested.

Since lesion diameter followed a skewed frequency distribution mean deviation from median was calculated as the measure of dispersion. The mean deviation from median recorded for Kalluvally was 0.32 where as it was only 0.24 for Cheriakanyakkadan indicating the high amount of somaclonal variation in the cultivar Kalluvally as compared to Cheriakanyakkadan.

4.11.3 Effect of duration in culture on somaclonal variation

The clones derived from a single callus culture were evaluated for the intensity of lesion development. The lesion development in regenerants which had passed seven to ten subculture cycles was evaluated to know the influence of duration in culture on somaclonal variation. Longer the culture period, higher was the variability observed. The range of variation was found to increase at the elapse of each subculture cycle (Table 49). The range of variation was 88 per cent more in regenerants which had passed ten subculture cycles as compared to regenerants which had passed seven subculture cycles.

Fig.14 Somaclonal variation in electrolyte leakage

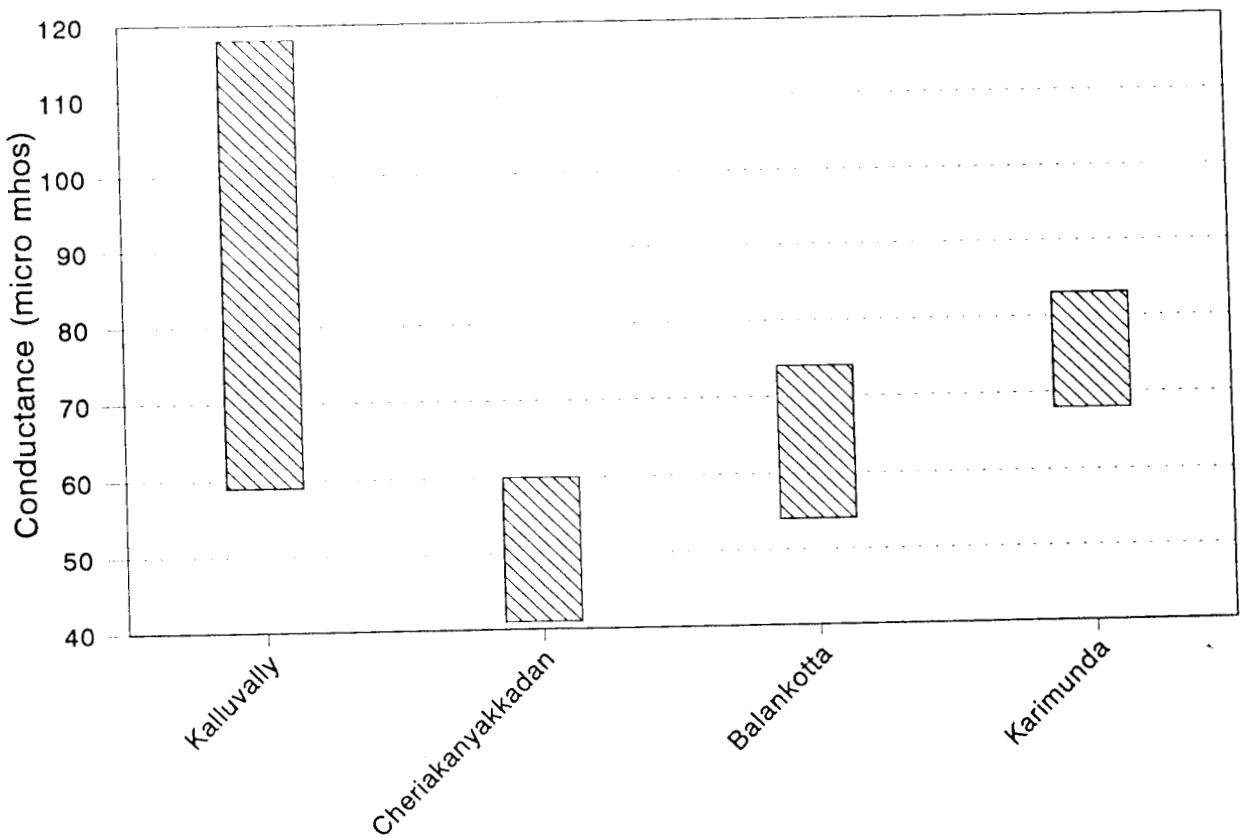


Table 48. Somaclonal variation in lesion development in black pepper cultivars Kalluvally and Cheriakanyakkadan

Sl. No.	Class based on lesion diameter in cm	Cultivars			
		Kalluvally		Cheriakanyakkadan	
		% of plants	LDS	% of plants	LDS
1	< 0.5	3.33	3.33	7.83	7.83
2	0.5-1.0	30.00	60.00	64.71	129.42
3	1.1-1.5	43.33	129.99	21.57	64.71
4	1.6-2.0	23.33	93.32	5.88	23.52
Total LDS			286.64		225.48

No. of calliclones derived from single seedling

Cheriakanyakkadan - 51
Kalluvally - 30

Mean deviation from median

Kalluvally - 0.32
Cheriakanyakkadan - 0.24

Table 49. Effect of duration in culture on somaclonal variation in lesion development (cv. Kalluvally)

Culture number	No. of subcultures elapsed	No. of regenerants	Lesion diameter (cm)		Range of variation
			Lowest	Highest	
DL 99	7	8	0.83	1.97	1.14
DL 99	8	6	0.84	2.23	1.39
DL 99	9	15	0.50	1.89	1.39
DL 99	10	51	0.35	2.50	2.15

Discussion

DISCUSSION

Black pepper (*Piper nigrum* L.), the king of spices, is now plagued with the dreadful *Phytophthora* foot rot disease. The disease, which is prevalent in all pepper growing tracts is reported to be the most destructive one and takes a heavy toll of the crop. Integrated disease management involving chemical, cultural and biological methods besides host resistance is perhaps the most ideal strategy to combat the disease (Sarma *et al.*, 1988). The conventional breeding programmes so far carried out could hardly trace out or evolve any genotype resistant to this devastating disease. Therefore, *in vitro* culture induced genetic variation (somaclonal variation) constitute an important source of variability for the improvement of this crop.

Sarma and Ramadasan (1990) stressed the importance of biotechnological approaches to induce resistance in black pepper. They reported the possibilities of cell/calli screening using toxins of *P. capsici* and protoplast fusion of *P. colubrinum* and *P. nigrum* to evolve hybrid lines of multiple resistance and productivity.

The present studies were focussed on the exploitation of somaclonal variation for screening for resistance/tolerance to *Phytophthora* foot rot disease in black pepper. The results of the various studies carried out are discussed in this chapter.

5.1 Preparation and assay of culture filtrate

In vitro selection for disease resistance using culture filtrates/purified toxins of pathogens has received much attention now-a-days.

In the present investigation *P. capsici*, the causal organism of *Phytophthora* foot rot disease in black pepper was found to produce toxic metabolite(s) *in vitro*, as confirmed by leaf puncture bioassay. The concentrated culture filtrate from shake cultures of *P. capsici* was found to produce the same symptoms as natural and artificial infection on detached leaves of the cultivar Karimunda, thereby fulfilling the criteria prescribed for a phytotoxin by Graniti (1972) (Table 1). *In vitro* production of toxic metabolite(s) by *P. capsici*, was also reported from the studies conducted by Lee (1973), CPCRI (1979) and Vilasini (1982).

The production of toxin by different organisms varied widely according to the culture conditions, nutrients, nutrient sources, pH of the medium, temperature and light conditions (Shaw, 1981). In the present investigation toxic metabolite(s) was found to be accumulated only in shake cultures. Production of *in vitro* phytotoxin in shake cultures of *P. nicotianae* var. *parasitica* was reported by Ballio *et al.* (1972). The principal advantage of giving agitation is that fermentation will proceed at a faster rate, probably because it allows more rapid diffusion of oxygen through the culture medium and media constituents (Shaw, 1981).

The toxic metabolite(s) accumulated in culture filtrates of *P. capsici* was found to withstand autoclaving, suggesting the thermostable nature of the metabolite(s) (Table 2). Heat stability of the toxic metabolites produced by *P. megasperma* var. *sojae* was reported by Paxton (1972) and *P. cinnamomi*, *P. palmivora* and *P. megasperma* var. *sojae* by Keen (1975).

In the present study, CCF was found to induce symptoms in all the crops in which it was inoculated showing that the toxin produced by *P. capsici* is non-specific (Table 4). The non-specific nature of the toxin produced by *P. capsici* was also reported by Sarma *et al.* (1991).

According to Yoder (1981) multiple assays may be helpful to draw more informations about the toxin. Hence in the present study electrolyte leakage assay of CCF was also conducted in addition to leaf puncture bioassay.

A common effect of toxin action is plasmalemma damage and for some toxins the plasmalemma has been suggested as the primary site of action although there is no conclusive evidence for this in any case (Rudolph, 1976; Scheffer, 1976 and Yoder, 1980). Changes in plasmalemma characteristics occur quickly after exposure to toxin. In several species, loss of electrolytes occurs in tissues infected with the fungus or treated with its toxin (Wheeler and Black, 1963; Samadar and Scheffer, 1968) or with its culture filtrates (Collins and Scheffer, 1958). Damann *et al.* (1974) showed that an electrolyte leakage assay for *Helminthosporium victoriae* toxin is as sensitive as the standard seedling root growth assay (Luke and Wheeler, 1955). It has a more precise dose response relationship which is highly reproducible if procedures are standardised and the results can be obtained quickly. Hence the increase in conductivity of leachates of host tissues when treated with pathogenic toxins has been used as a sensitive assay for many *in vitro* studies of pathogenicity.

In the present investigation, CCF induced quick electrolyte leakage from both the leaves and calluses suggesting the possibility of plasmalemma as the site of action of toxic metabolite(s) (Table 5a, 5b). For an efficient *in vitro* selection system

the screening agent should act at the level where selection is carried out in the same manner as it does on the whole plants. Since CCF induced quick electrolyte leakage from the calluses as in leaf tissues its activity at callus/cellular level was confirmed. Cellular level activity of *Helminthosporium oryzae* toxin and cercosporin in rice was reported by Vidyasekharan *et al.* (1990) and Batchvarova *et al.* (1992) respectively.

Electrolyte leakage from leaves of *P. nigrum* cultivars and *P. colubrinum* showed that Panniyur-1 is most susceptible to CCF followed by Karimunda (Table 6a). Kalluvally and Cheriakanyakkadan are showing tolerance to CCF. The tolerance of Kalluvally and Cheriakanyakkadan to *P. capsici* and high susceptibility of Panniyur-1 and Karimunda to the fungus was reported by Kueh and Khew (1980b) and Sarma *et al.* (1982). So the cultivars showing the same level of tolerance to *P. capsici* are exhibiting the same type of tolerance to CCF, suggesting the possibility of utilising CCF for *in vitro* screening.

Electrolyte leakage from calli of *P. nigrum* cultivars and *P. colubrinum* exhibited the same trend as in the case of leaf tissue (Table 6b). The similarity in response in leaves and calluses to *Pyricularia oryzae* toxin was observed in rice by Velazhahan *et al.* (1993). The result also indicated that the level of tolerance observed to *P. capsici* in the whole plant level in black pepper was expressed at the same level in the callus phase also. The similarity in disease reaction between calluses and whole plants was reported in eucalyptus to *P. cinnamomi* (Mc Comb *et al.*, 1987), in alfalfa to *P. megasperma* (Miller *et al.*, 1984) and in pines to *P. cinnamomi* (Jang and Tainter, 1990). This response suggested the potential use of callus cultures for *in vitro* screening and for understanding the host-pathogen interactions at cellular level.

5.2 *In vitro* screening of calli using concentrated culture filtrate

Concentrated culture filtrate induced callus necrosis on susceptible calli and it was not possible to revive the necrotic calli on a non-toxic medium. Callus necrosis was taken as the index of assessing the effect of toxic metabolites in almost all *in vitro* screening studies.

The percentage of necrotic calli exhibited at different concentrations of CCF differed significantly in the different cultivars studied (Table 8a, 8b). Higher the level of CCF in the media, higher was the callus necrosis. The highest callus necrosis was observed in those cultivars showing highest electrolyte leakage values. So also, the percentage of callus necrosis was in accordance with the resistance/susceptability reaction reported for the cultivars to *P. capsici* at whole plant level. Panniyur-1 showed the highest callus necrosis followed by Karimunda, while Kalluvally and Cheriakanyakkadan showed lower necrosis. Similar difference in response of cultured cells of host cultivars that have different levels of resistance to the pathogen was reported in studies with culture filtrates of *Xanthomonas campestris* pv. *pruni* in peach (Hammerschlag, 1984), *Alternaria solani* (Handa *et al.*, 1982) and *Verticilium* sp. (Hartman and Secor, 1985) in potato and *Pseudomonas syringae* pv. *phaseolicola* in beans (Hartman *et al.*, 1985).

5.3. Effect of concentrated culture filtrate at various stages of development of calli

Once the calli survived in the CCF added medium, CCF was not inhibiting further growth of the surviving calli (Table 9a and 9b). However, prolonged duration of selection with CCF (for a period of 3 months) inhibited the

regeneration potential of the calli totally (Table 10). Poor regeneration of selected callus/cell lines against toxins was reported to be one of the major problems in *in vitro* selection system (Brar and Vidyasekharan, 1990).

Arcioni *et al.* (1987) observed reduced regeneration potential for culture filtrate resistant cells of alfalfa when screened against *Fusarium oxysporum* f. sp. *medicaginis*, where in the lines used were characterized by high regeneration capability. They also observed that continuous culture in culture filtrate added medium for seven months completely arrested the plant regeneration potential. Even very low concentration of toxin was found to inhibit regeneration in two of the callus lines in lucerne(Latunde Dada and Lucas, 1988). Wolf and Earle (1990) reported that inhibition of regeneration in resistant maize calli to *Helminthosporum carbonum* was due to harmful metabolites produced in the surroundings of necrotic cells. Vidyasekharan *et al.* (1990) obtained no regeneration from resistant rice callus when screening was done for brown eye spot disease using *Helminthosporum oryzae* toxin.

However, shoot regeneration was observed in calli of axenic seedlings of the cultivar Karimunda in CCF added medium (Table 10). This might be due to the early induction of shoots observed in that cultivar. Three to five days after inoculation in the CCF added medium calli of Karimunda put forth shoots since they were already in the regeneration pathway. So CCF might not have influenced the regeneration in Karimunda.

The toxic metabolite(s) in the culture filtrate was not found to inhibit further proliferation of shoots and shoot growth in the already regenerated cultures (Table 11 and 12). However, CCF had its influence on root growth (Table 13). The number of roots showed no significant variation but the length and thickness of roots

showed significant variation in CCF added medium. The roots were found to be thinner and longer in CCF added medium compared to the control.

Detailed symptomatology of Phytophthora foot rot disease in black pepper by Mammootty (1978) and Sarma and Nambiar (1982) showed that root and collar regions of the vine are more vulnerable to the attack by the pathogen. In the present *in vitro* studies also, out of the various stages of development observed, the CCF had its maximum effect on root growth reducing the thickness of roots significantly.

As pointed out by Pegg (1976) and Yoder (1980) culture filtrates of fungi are rich in secondary metabolites, growth inhibiting and stimulating substances. Since CCF was giving better proliferation of shoots the presence of other secondary metabolites in the filtrate cannot be ruled out (Table 11).

5.4 Direct selection of calli by different screening methods

The duration of selection pressure ie. the number of selection cycles used, can affect the outcome of *in vitro* selection experiments. Various selection systems such as direct selection, prolonged duration of selection, discontinuous selection, selection with habituation by applying progressively higher concentrations of the selective agent in successive cycles had been reported in *in vitro* selection studies.

In the present investigation, since prolonged duration of selection in CCF added medium was arresting the regeneration potential totally, direct selection scheme was adopted. Three direct selection methods viz. growing in CCF incorporated medium (Method 1), shaking in CCF added liquid medium (Method 2) and double layer culture technique (Method 3) were used for screening the calli. Of

the different methods, first one was used in many *in vitro* screening studies, as reported by Carlson, 1973; Ling *et al.*, 1985; Larkin and Scow Croft, 1983; Pauly *et al.*, 1987; Gengenbach and Green, 1975; Behnke, 1979; Shahin and Spivey, 1986; Vidyasekharan *et al.*, 1990. Vidyasekharan *et al.* (1990) used toxin shaking (Method 2) to regenerate rice calluses resistant to brown eye spot disease. Ahmed *et al.* (1991) regenerated wheat plants showing more tolerance to *Fusarium* spp. than the original cultivars adopting the double layer culture technique (Method 3) for screening the calli.

In all the three selection methods Kalluvally, which was showing low electrolyte leakage and in which field tolerance to the disease was reported was found to exhibit the lowest callus necrosis followed by Cheriakanyakkadan (Table 8b, 18, 22). Panniyur-1 which showed the highest electrolyte leakage values and which was reported to be susceptible to the disease showed the highest callus necrosis.

Out of the three methods tried, regeneration and survival of healthy cultures for all the cultivars except Panniyur-1 was achieved in the first method, while only Kalluvally was found to give healthy cultures from the 2nd and 3rd method of screening (Table 14, 19, 22). The better performance of Kalluvally might be due to its inherent ability to withstand all stress conditions (Ravindran and Nair, 1983).

The effect of callus screening on all stages of development of cultures was studied in detail (Tables 14 to 24). The regeneration of shoots from calli, further proliferation of shoots and recovery of rootable shoots were not influenced by the callus screening methods tried. But the root growth was found to be influenced by the method of screening. The regenerations produced from screened

calli by method 1 registered significantly lower number of thinner roots as compared to the regenerants from unscreened calli (Table 17). Comparison of the anatomy of roots from screened and unscreened calli derived clones showed that conducting tissues were more affected (Plates 12a to 12d). Vascular tissue discolouration and collapse of conducting tissues in *P. capsici* affected black pepper roots were reported by Mammooty (1978). It could be presumed that the major portion of the toxic metabolites absorbed by the callus cells was found expressed in the rooting stage and that might be the reason for the thinner roots observed in screened cultures. With regard to the thickness of roots in cultures screened by the other methods, no significant differences were observed between the screened and unscreened ones (Table 21 and 24). This might be due to the lesser amount of toxic metabolites absorbed by the calli and due to the lesser contact of the calli with CCF. In method 2 the quantity of CCF absorbed by the calli during the 48 h shaking will only be translocated while in method 3 the quantity of toxic metabolite(s) diffused in the upper layer will only be absorbed by the callus cells. So the quantity of toxic metabolites absorbed and translocated might be less in method 2 and 3 compared to method 1 in which the cells were in continuous contact with CCF for a period of 21 days.

5.5 *In vitro* induction of mutation by gamma irradiation

When the effect of gamma irradiation on callus growth was studied the cultivars were found to differ significantly (Table 25, 26 and 27). Kalluvally could withstand higher doses of gamma irradiation compared to the other cultivars. This might be due to its inherent ability to withstand the stress conditions and due to the influence of genotypes. The effect of genotype in the response to gamma irradiation was observed by Pinet-Leblay *et al.* (1992) in pine.

At lower doses ranging from 17.5 Gy to 25 Gy Kalluvally was found to give better growth compared to non-irradiated control. Slight stimulation of growth at lower doses of irradiation was reported in bean tissue cultures by Bajaj *et al.* (1968).

In all the cultivars growth inhibition was found in higher doses (40 and 50 Gy) compared to the non-irradiated control. The colour and nature of calli were also found to vary at higher doses of irradiation (Table 27). This can be attributed to the effect of gamma irradiation. Bajaj *et al.* (1970) stated that gamma irradiation in general caused inhibition of tissue growth along with failure of RNA synthesis and subsequently protein.

In the present study the regeneration potential of irradiated calli was found to be very low even for calli induced from *in vitro* seedling explants (Table 28). This might be due to the low radio sensitivity of the calli compared to the seeds, seedlings or any other organized tissue (Venketeswaran and Partanen, 1966; Bajaj, 1970 and Rao *et al.*, 1976). Venketeswaran and Partanen (1966) observed surviving tobacco calli even after subjecting them to a gamma dose of 20,800R while the growth of apical meristem was stopped at a dose of 5200R. Stimulation of callus growth and suppression of shoot regeneration in doses above 2 kR in mustard cotyledon explants of the var. Rai-5 was reported by George and Rao (1980). As in the case of present study George and Rao (1980) got small, poorly developed shoots from cotyledon cultures of mustard. Inhibition of shoot regeneration at a higher dose of 5 kR and stimulation at lower dose of 0.2 and 1 kR was reported in *Datura innoxia* by Jain *et al.* (1984). The decrease in plant regeneration capacity was correlated with a change in tissue composition of the

treated maize callus by Moustafa *et al.* (1989) and they quantified the regeneration potential of the calli by PR assay.

When irradiated calli were used for screening by growing in CCF added medium no better performance in growth and regeneration was observed (Table 29). Regeneration was not achieved in any of the cultivars except Kalluvally where 5.55 per cent of cultures put forth very weak chlorotic shoots.

5.6 Partial purification of the culture filtrate of *Phytophthora capsici*

The results reported here demonstrated that the toxic metabolite(s) present in the culture filtrate of *P. capsici* could not be separated by organic solvent fractionation and the toxic metabolite(s) were present in aqueous fraction of the filtrate (Table 30). The presence of toxic metabolites in aqueous fraction of the filtrate of *P. megasperma* var. *sojae* causing *Phytophthora* root and stem rot of soyabeans was reported by Paxton (1972). The presence of phytotoxic metabolites in aqueous fractions in other species of *Phytophthora* viz. *P. cinnamomi, P. palmivora* and *P. megasperma* var. *sojae* was also reported by Keen *et al.* (1975).

Since organic solvent fractionation was found unsuccessful, separation of toxic metabolite(s) using ion exchange chromatography was attempted. Toxic metabolite(s) present in the aqueous fractions could be separated using Dowex 1 and Dowex 50 ion exchangers. The fractions separated through Dowex 1 and 50 when subjected to leaf puncture bioassay, it was found that the nature of spots formed for the fractions collected from both the columns were the same. But the intensity of spot development was more in fraction collected from Dowex 50 (Plate 18). This showed that the concentration of the toxic fraction collected from Dowex 1 was achieved in Dowex 50. The CCF and the fractions collected from Dowex 1 and 50

were showing the same symptoms which indicated the selective inhibition of the toxic metabolite(s) at the specific site and the effectiveness of the inhibitor.

However, much more effort is needed to chemically characterise the toxin and to quantify it. The thermostable nature of the toxic metabolite(s), its presence in aqueous fraction and its specific activity whether purified or not give the assumption that the toxic metabolite(s) present in culture filtrate of *P. capsici* may be mycolaminaran (Water soluble ß 1-3 glucans) as reported in other *Phytophthora* spp. by Keen *et al.* (1975).

5.7 Production of somaclones without *in vitro* callus screening and screening the regenerants at whole plant level

Calliclones were produced in all the five cultivars without applying *in vitro* selection pressure with toxic metabolite(s) of *P. capsici*. Screening the calli clones for *Phytophthora* foot rot resistance/tolerance was done at whole plant level using the conventional methods of disease screening.

Comparative performance of the five cultivars in callus induction, shoot regeneration, shoot proliferation, recovery of rootable shoots, rooting and root growth was studied (Table 31 to 36). The five cultivars differed significantly in days taken for callusing, percentage of callusing and callus proliferation (Table 31a, 31b, 31c and Fig.6). The number of days taken for shoot induction and the number of cultures showing regeneration of shoots also differed significantly in the cultivars studied (Table 32). Kalluvally registered the highest recovery of rootable shoots in all subcultures followed by Cheriakanyakkadan (Table 35). The cultivars studied showed highly significant variation in root number and root thickness (Table 36). Cheriakanyakkadan registered higher number of roots with higher root thickness.

The influence of genotypes in the *in vitro* response had been observed by several workers (Tabata and Motoyoshi, 1965; Parfitt and Almehdi, 1986; Miller and Ferree, 1988; Reghunath, 1989; Reed, 1990; Dulieu, 1991; Kristiansen, 1992; Brandt, 1992; Rietveld *et al.*, 1993, Brandt, 1994 and Das *et al.*, 1995a and b and Philip *et al.*, 1995). The reason for the genotypic difference in *in vitro* performances can be attributed to the difference in endogenous cytokinin and/or auxin in the genotype (Ahokas, 1985; Looney *et al.*, 1988; Maldiney *et al.*, 1986; Mapelli and Lombardi, 1982; Sossountzov *et al.*, 1988) as well as differences in *in vitro* uptake of exogenous cytokinins (Marino, 1988). The differences in *in vitro* uptake of exogenous cytokinins lead to differences in the endogenous balance of auxin and cytokinin (Lane *et al.*, 1982; Von Arnold, 1984; Bergman *et al.*, 1986; Von Arnold and Tillerberg, 1987; Alvarez *et al.*, 1989 and Gronroos *et al.*, 1989) and hence the difference in response.

5.8 Planting out, hardening and final survival

The effect of season, callus screening and the influence of cultivars on final survival of plantlets were studied (Tables 37 to 39).

It was found that the mortality of plants during hardening showed a positive correlation with relative humidity (Appendix-IV). This is in contrast with the reports made by several authors that the longest simple factor resulting in the poor post transfer growth and survival of *in vitro* raised plants is the drop in relative humidity from near to 100 per cent in the culture vessels to much lower values in the glass house or in the field (Grout and Aston, 1977; Wetzstein and Sommer, 1982). Compared to other crops black pepper requires less humidity in the hardening period. If the humidity is high there is rotting of leaves, stem and collar region of the plantlets. In this investigation majority of the plantlets were planted out from

June to August (Appendix-II) during which period the atmospheric relative humidity was high (86-87%). So during this period the plantlets were not provided with humid cap. When the atmospheric relative humidity was low the plantlets were given humid cap provided with holes so as to reduce the excess humidity. The final survival of the plantlets was however positively correlated with rainfall, number of rainy days and relative humidity and negatively to sunshine hours (Table 37, Appendix-III and IV).

The plantlets regenerated from screened calli showed high mortality and low final survival as compared to plantlets from unscreened calli (Table 38). This might be due to the reduced root thickness and poor development of conducting tissues observed in the screened plantlets as compared to the unscreened ones (Table 17). Of the different cultivars studied Karimunda was showing the highest reduction in root thickness and the maximum difference in final survival between screened and unscreened plantlets. Since the reduction in root thickness was not pronounced in plantlets derived from screened calli by method 3, the survival rate was also high compared to method 1 (Appendix-V). So the thickness of roots and development of conducting tissues had got its influence on final survival of plantlets.

The influence of cultivars on final survival when compared, it was found that the highest final survival of 62.76 per cent was recorded for Cheria-kanyakkadan followed by Karimunda (54.24%) (Table 39). This might be due to the large number of thick healthy roots present in these cultivars compared to others.

5.9 Screening the calliclones for tolerance/resistance to *Phytophthora capsici*

In the three different methods of screening tried in calli clones viz. natural screening, screening by electrolyte leakage method and screening by artificial

inoculation of culture disc of *P. capsici*, the performance of plantlets derived from screened calli was found to be poor compared to unscreened ones.

In natural screening, since all the plants took infection and wilted completely, it could be presumed that none of the regenerated calliclones were resistant to the disease.

Further, the screened calli derived clones were the first to take infection although plantlets from both the sources took infection and wilted completely (Table 40 and 41). In screening by electrolyte leakage method, leaf tissues from screened calliclones showed higher leakage values as compared to unscreened ones (Table 42). Similarly in screening by artificial inoculation of culture disc of *P. capsici* the overall lesion development score when compared between the screened and unscreened group, irrespective of the cultivars, the regenerants from screened calli registered 11.10 per cent lesion score (Table 43).

The results of the present study also demonstrated the differential expression of CCF resistance between cultured cells and regenerated whole plants. In culture, the calli induced from the cultivar Kalluvally were showing the lowest callus necrosis, lowest leakage of electrolytes and thereby highest resistance to CCF. But in the regenerated calliclones, Kalluvally was found to be more susceptible to the disease compared to Cheriakanyakkadan (Table 45).

Although CCF showed the same response as that of *P. capsici* in resistance/susceptibility reaction in the callus phase of the five cultivars, the resistance/susceptibility reaction was found different in the whole plant level ie. in the regenerated calliclones. In the callus phase many of the calli of the cultivars studied showed resistance to CCF but the regenerants produced from the resistant

calli showed susceptibility to CCF as indicated by the high leakage of electrolytes from leaf tissues of the screened plantlets as compared to the unscreened ones.

Differential expression of toxin resistance in the cultured cells and whole plants was reported to be one of the major drawbacks of *in vitro* screening system (Brettel and Ingram, 1979; Daub, 1986; Brar and Vidyasekharan, 1990; vanden Bulk, 1991). Lack of correlation between *in vitro* response to toxin and response of the whole plants to infection has been reported by Kumashiro (1983); Mac Donald and Ingram (1986); Vardi *et al.* (1986); Newsholme *et al.* (1989) and Meghegneau and Branchard (1991).

As pointed out by Goodman *et al.* (1986) that the role of many phytotoxic compounds isolated from culture filtrates of many pathogenic fungi and bacteria in pathogenesis has not been elucidated, the same is true in the case of toxic metabolites produced by *P. capsici*. The toxic metabolite(s) produced by *P. capsici* are known to be non-specific which are considered only as secondary determinants of the disease or virulent factors ie. compounds which increase the extent of disease symptoms but are not involved in the primary interaction that determines compatibility. Consequently plants resistant to these toxins probably do not show complete resistance to the pathogen.

Once the toxic metabolite(s) is absorbed by the cells/calli, the site and mode of action of the metabolite(s), its translocation, its conversion into other metabolically active compounds, its actual role in disease development etc. are not clearly understood.

In the present study, since the toxic metabolite(s) present the aqueous fraction of the filtrate was found difficult to separate in large quantities and to use in

in vitro screening studies, crude culture filtrate of the fungus was used as the screening agent. By utilising culture filtrate as the screening agent isolation of disease tolerant/resistant lines was reported by several workers (Behnke, 1979; Arcioni *et al.*, 1987; and Evenor *et al.*, 1994). As pointed out by Yoder (1983) that crude culture filtrates would not necessarily be expected to select plants resistant to diseases since growth media that have been colonized by microorganisms (whether pathogenic or not) contain many secondary metabolites which in combination can be phytotoxic but have nothing to do with disease development. Pegg (1976) and Yoder (1980) reported the presence of growth inhibiting and growth stimulating substances in culture filtrates of fungi/bacteria along with secondary metabolites. There are many substances in the culture filtrates like cytokinins (Johnson and Trione, 1974), auxins (Gruen, 1959; Epstein and Miles, 1967) and gibberellic acid (Brian *et al.*, 1954), several amino acids, high and low molecular weight phytotoxins as in culture filtrate of *P. citrophthora* (Breiman and Barash, 1981).

It was observed from the present studies that the toxic metabolite(s) produced by *P. capsici* was found to withstand autoclaving. Perusal of the literature, results of the trials conducted for purification of toxic metabolite(s) and the more number of starch grains in screened calli derived roots showed that the toxic metabolites present in *P. capsici* might be mycolaminarans (ß 1-3 glucans) as reported in other *Phytophthora* spp. by Keen *et al.* (1975). Break down of the constituents or reaction among different constituents are inevitable during the process of autoclaving (Van Bragt *et al.*, 1971). These polysaccharides present in toxic metabolite(s) of *P. capsici* along with sucrose added in the media might have also been converted to phytotoxic compounds during autoclaving. A variety of products are formed when carbohydrates are subjected to high temperature treatment (Theander and Nelson, 1989). Some of these products have been identified using

fructose as a model carbohydrate (Shaw *et al.*, 1967, 1968). The main component is 5(hydroxy methyl)-2 furaldehyde which is known to be biologically toxic (Moye, 1964; Weatherhead *et al.*, 1978). Other toxic compounds derived from monomeric saccharides are phenolics (Suorotti, 1983). Since the leaf puncture bioassay with autoclaved CCF gave better development of symptoms, the formation of interaction compounds during autoclaving can not be ruled out.

So the phytotoxicity observed for the calluses during *in vitro* screening was not necessarily due to the toxic metabolites alone but due to secondary metabolites present in the filtrate and due to phytotoxic substances formed during the process of autoclaving. The presence of auxin/cytokinin like substances or any other substances which affect the auxin-cytokinin balance in the culture filtrate of *P. capsici* also can not be ruled out since the CCF added medium was giving better proliferation of shoots compared to the control. The presence of auxin like substances in the culture filtrate of *P. citrophthora* was reported by Vardi *et al.* (1986) and better regeneration of shoots and roots in the presence of non-specific bacterial blight phytotoxin (Syringomycin) in wheat was reported by Pauly *et al.* (1987).

A thorough knowledge on the mode of action and properties of the toxin involved is also important for the success of *in vitro* screening system. A recent investigation on the reaction of tissue cultures of potato to Vd toxin produced by *Verticillium dahliae* showed that the protoplasts of a susceptible genotype were virtually unaffected where as suspension cells were affected (Nachmias *et al.*, 1990). Since the toxin acts on K^- and Na^- ion transport systems in plasma membranes it was suggested that the isotonic conditions during the protoplast assay interferred with the effect of the toxin on the plasma membrane. In the study the calli were also

unaffected except for the cells in direct contact with the toxic medium. The lack of xylem vessels in callus cultures which hinder the exposure of the inner cells to the toxin was reported to be the reason for this response. The importance of the mode of action and properties of toxins was also demonstrated by the work of Daub (1984) who was unsuccessful in her efforts to select cells of tobacco and sugar beet resistant to the non-specific toxin cercosporin.

An insight into the biochemical basis of host-pathogen interactions is also of great significance. Studies on the biochemical basis of the resistance/susceptibility reaction to *Phytophthora* foot rot disease in black pepper and related species will be helpful in the development of an evaluation system for *in vitro* disease resistance.

The lack of correlation in disease reaction between the cultured cells and regenerants obtained in the present studies might be due to non-specific toxic metabolite(s) employed in the screening work whose actual role in pathogenesis is not well understood. Since correlation of disease reaction was not observed in cultured cells and regenerated whole plants in the study, it can be presumed that the toxic component in the filtrate might have only a limited role in pathogenesis. The interference of other substances found in the culture filtrate which had no role in disease development also cannot be ruled out. It is not known that the toxic metabolite(s) accumulated in the culture filtrate under *in vitro* conditions is identical in terms of quantity and quality to toxins secreted during actual pathogenesis. So it may not be possible to predict the quantity of the toxic metabolite(s) employed for screening the calli - whether the quantity of toxic metabolite(s) given was high/low or sufficient. The deficient knowledge on the site and mode of action of toxic metabolite(s), their interaction with host cells and the basic genetic and biochemical aspects of disease reaction also added to the differential expression.

The most striking feature of the present investigation is that eventhough the screened calliclones showed susceptibility, the clones from unscreened calli showed better tolerance to *P. capsici*.

In natural screening for resistance to *P. capsici* although none of the clones were found to be totally resistant, clones originated from unscreened calli were found to take infection late. In screening by electrolyte leakage method the unscreened calli derived clones from all cultivars except Kalluvally registered significantly lower electrolyte leakage values as compared to the screened ones. Moreover, the unscreened calliclones from Cheriakanyakkadan registered lower electrolyte leakage values than calliclones from all other cultivars showing its more tolerance to the CCF and there by to the fungus. The differential response observed in Kalluvally might be due to the high clone to clone variability observed in the cultivar Kalluvally. In the third method of screening by artificial inoculation of culture disc of *P. capsici*, the intensity of lesion development was less in unscreened calli derived clones as compared to clones from screened calli. Out of 142 clones of the unscreened group observed for lesion development 49.30 per cent showed lesion diameter up to 1.0 cm while only 30.17 per cent of the clones from screened source showed lesion diameter up to 1.0 cm. The better performance of calliclones produced without *in vitro* screening might be due to the high amount of culture induced variability.

Therefore, the clones derived from callus cultures of a single seedling of four black pepper cultivars viz. Kalluvally, Cheriakanyakkadan, Balankotta and Karimunda were studied in detail to know the extent of somaclonal variation.

The high variability in lesion development and electrolyte leakage values observed among the calliclones within a cultivar clearly demonstrated the existence of somaclonal variation in black pepper.

The amount of somaclonal variation exhibited is high in the cultivar Kalluvally as compared to other cultivars. The high amount of variability in electrolyte leakage values (Table 47) and high variability in lesion diameter (Table 48) indicated the high amount of somaclonal variation in the cultivar Kalluvally. The increased variation observed in Kalluvally may be due to the effect of genotype. Daub and Jenns (1989) reported that the genotype of the parents decided the variability in tobacco somaclones. Another reason for the high rate of somaclonal variation in Kalluvally may be the high proliferation rate observed in the cultivar as compared to others. As pointed out by Smith and Drew (1990) cultures proliferated at excessive rates show more variation than those grown at moderate rates. The possibility of nuclear irregularities in a highly multiplying culture also should be emphasised in this context.

In the present study it was observed that longer the period in culture, higher was the range of variation observed in lesion development in the somaclones (Table 49). This is in confirmity with the observation made by Skirvin *et al.* (1994) that the length of time that a culture has been maintained *in vitro* is the most important factor involved in inducing somaclonal variation.

Another significant achievement is the more number of tolerant plants in the cultivar Cheriakanyakkadan as compared to Kalluvally. Cheriakanyakkadan was found to give 72.54 per cent of plants with up to 1.00 cm lesion diameter while only 33.33 per cent of somaclones in the cultivar Kalluvally came with in the group.

The use of culture induced variability as such (somaclonal variation) without resorting to *in vitro* selection was demonstrated by several workers in disease resistance breeding (Heinz and Mee, 1969; Krishnamurthy and Tlaskal, 1974; Bidney and Shepared, 1981; Larkin and Scowcroft, 1983 and Daub and Jenns, 1989).

Several investigators compared the results obtained from unselected cultures to those obtained from cultures screened with toxins or pathogens (Brettel *et al.*, 1980; Sacristan, 1982; Hartman, 1983; Hartman *et al*, 1984; Ling *et al.*, 1985 and Latunde Dada and Lucas, 1988). In all these cases, they were able to isolate resistant plants in the absence of selection.

By exploiting the somaclonal variation as such dominant and homozygous recessive traits can directly be selected. If the regenerants are heterozygous for a desired character, recessive traits can be selected in the progenies of the regenerants (van den Bulk, 1991). In the case of asexually propagated species transmission of the variant traits through at least two successive clonal propagation cycles provides reasonable assurety of a true genetic base (Scowcroft, 1984).

Based on the present investigations, chances of getting tolerant somaclonal variants to *Phytophthora* foot rot in black pepper through callus mediated organogenesis is confirmed. So the future thrust should be given for the production of variant plants using all the cell culture techniques and screening the whole plants for resistance to *Phytophthora* foot rot. Eventhough, *in vitro* selection procedures may increase the efficiency of selection our deficient knowledge about the basic genetic and biochemical processes involved in host-pathogen interaction remains a major bottle neck.

The *in vitro* screening system standardised using CCF in the present studies can however be used for screening an available germplasm of black pepper to *Phytophthora* foot rot tolerance, since tolerance at whole plants are transferred as such to the callus phase and CCF can be used in place of the fungus.

But for the production of disease resistant plants through *in vitro* screening system, studies regarding the actual role of non-specific toxin of *P. capsici* in disease development, the chemical characterisation of the toxin, the quantity of toxin produced in actual pathogenesis, the nature and quantity of toxin accumulated *in vitro* in liquid culture etc. needs thorough investigation.

The high clone to clone variability observed in Kalluvally and the tolerance level observed for Cheriakanyakkadan in the present investigation should be exploited in further studies.

However, a co-ordinated effort of tissue culturist, pathologist and biochemist is needed to achieve the final goal of resistance to *Phytophthora* foot rot disease in black pepper through cell and tissue culture techniques.

Summary

SUMMARY

Investigations were carried out on the exploitation of somaclonal variation for screening for resistance/tolerance to *Phytophthora* foot rot disease in black pepper, at the Plant Tissue Culture Laboratory of the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara. The salient findings of the study are summarised in this chapter.

1. Toxic metabolite(s) was found to be accumulated in the culture filtrate of *P. capsici*.

2. Leaf puncture bioassay of the concentrated culture filtrate gave necrotic spots which was quite typical of the natural and artificial infection of *P. capsici*.

3. The toxic metabolite(s) present in the culture filtrate of *P. capsici* was found to be non-specific and thermostable.

4. Concentrated culture filtrate induced quick electrolyte leakage from leaves and calluses. The pattern of electrolyte leakage from the leaves and calluses followed the same trend. The quick electrolyte leakage from calluses revealed the activity of CCF at callus/cellular level. The site of action of the toxic metabolite(s) may be the plasmalemma.

5. Since CCF showed the same type of disease reaction as that of *P. capsici*, CCF can be used in place of the fungus for screening studies.

6. The tolerance to *P. capsici* observed in the whole plant level was carried over as such, to the callus phase.

7. Concentrated culture filtrate induced electrolyte leakage even at 2.5 per cent v/v. At lower concentrations of CCF, Kalluvally and Cheriakanyakkadan were showing the same type of response.

8. Concentrated culture filtrate induced necrosis on susceptible calli. Callus necrosis was taken as the index for assessing the effect of toxic matabolite(s) in *in vitro* screening studies. It was not possible to revive the necrotic calli on a non-toxic medium.

9. The higher the concentration of CCF in the modified MS medium, the higher was the callus necrosis. Based on callus necrosis in different cultivars 7.5 per cent v/v of CCF was selected as the level for callus screening and for studying the effect of CCF at various stages of development. Once the calli survived in CCF incorporated medium, CCF was not inhibiting further growth of calli.

10. Prolonged duration of selection with CCF was found to inhibit shoot regeneration totally. CCF did not inhibit further proliferation of shoots and shoot growth in already regenerated cultures but the root growth in culture was found to be influenced by CCF. CCF added medium was found to give thinner roots as compared to the medium control.

11. In all the direct selection/screening methods attempted for callus screening, Kalluvally was found to give lowest callus necrosis and Panniyur-1 the highest.

12. The calli of all the cultivars except Panniyur-1 could be regenerated after screening by method 1. In callus screening by the 2nd and 3rd method, regenerants from Kalluvally alone could be planted out.

13. Direct callus screening was not found to influence the regeneration of shoots, further proliferation of shoots and recovery of rootable shoots.

14. The thickness of the roots as well as the number of roots produced were found to be influenced by direct callus screening (Method 1). The regenerants from screened calli registered significantly lower number of roots with reduced thickness as compared to the unscreened ones.

15. The roots regenerated from screened calli showed poorly developed xylem vessels, reduced layers of cortical cells, less number of root hairs and more cell inclusions especially starch grains. In contrast, the roots of normal plantlets were characterised by well developed xylem vessels, more layers of cortical cells, more number of root hairs and less starch grains in cells.

16. The calli of Kalluvally could withstand higher dose of gamma irradiation as compared to other cultivars. Lower doses of gamma irradiation ranging from 17.5-25 Gy was found to stimulate the callus growth in Kalluvally.

17. In all the cultivars, callus growth inhibition was found at higher doses of gamma irradiation viz. 40 Gy and 50 Gy. At higher doses of gamma irradiation the colour of the calli produced was light brown to brown and the nature of the calli was friable.

18. Evaluating the growth performance, the dose 30 Gy was fixed as the maximum dose of gamma irradiation that the calluses of different cultivars could withstand.

19. The regeneration potential of irradiated calli was found to be very low. Irradiated calli could not give any better response to *in vitro* screening.

20. The toxic metabolite(s) present in the culture filtrate of *P. capsici* could not be separated by organic solvent fractionation and the metabolite(s) were present in aqueous fraction. The toxic fraction present in the culture filtrate could be separated using ion exchangers like Dowex 1 and Dowex 50.

21. The callusing and callus growth was influenced by the source of explant. Explants from *in vitro* established cultures were found to give cent per cent callusing and highest callus index.

22. The five cultivars differed significantly with respect to callusing and callus growth. Kalluvally registered the highest callusing and callus index followed by Karimunda, Balankotta, Cheriakanyakkadan and Panniyur- 1. Early callusing was observed in Kalluvally while late callusing was observed in Panniyur-1. The percentage of callusing, callus index and days taken for callusing in different cultivars ranged from 80.49-97.40, 141.98-274.92 and 12-16.44 days respectively.

23. Regeneration could not be achieved in calli induced from mature leaf explants. Early and high regeneration was observed in calli induced from *in vitro* established cultures. Karimunda showed highest regeneration potential (68.51%) followed by Kalluvally (63.5%). Panniyur-1 registered the lowest regeneration (33.33%). Early induction of shoots was observed in Karimunda (32.33 days) while late induction was observed in Panniyur-1 (58.33 days). Other cultivars took 42.33-45.83 days for shoot induction.

24. Big culture tubes of 380 x 200 mm were the best for getting highest proliferation of shoots. The highest proliferation of shoots was observed in Kalluvally followed by Karimunda and the lowest in Panniyur-1. The number of shoots proliferated in 3rd subculture was 18.16, 16.58 and 3.91 respectively in the cultivars.

25. Kalluvally registered the highest recovery of rootable shoots followed by Cheriakanyakkadan. Panniyur-1 recorded the lowest recovery of rootable shoots followed by Karimunda. The number of shoots recovered in 6th subculture was 12.00, 10.25, 2.08 and 5.00 respectively in the cultivars.

26. All the cultivars recorded cent percent rooting. Panniyur-1 took 15 days for the root initials to appear while all the other cultivars took 12.75 to 13.83 days. The cultivars showed highly significant variation in root number. The highest root number was recorded by Cheriakanyakkadan (17.66) closely followed by Kalluvally (15.83). The lowest root number was observed in Panniyur-1 (8.75). The highest root length (3.04 cm) was observed in Cheriakanyakkadan and the lowest in Panniyur-1 (2.19 cm). The mean root thickness in different cultivars varied significantly and ranged from 0.9 to 1.27 mm. The highest root thickness was observed in Cheriakanyakkadan followed by Karimunda and the lowest in Panniyur-1.

27. A positive correlation with relative humidity and mortality of plants was observed during and after hardening. The final survival of the plantlets however was positively correlated with rainfall, number of rainy days and relative humidity and negatively to sunshine hours.

28. The plantlets regenerated from screened calli showed low final survival as compared to plantlets from unscreened calli. When cultivars were compared, the highest final survival of plantlets was observed in Cheriakanyakkadan (62.76%) followed by Karimunda (54.24%), Kalluvally (46.49%), Panniyur-1 (46.15%) and Balankotta (34.78%).

29. None of the regenerated calliclones were found to be resistant to *P. capsici* in natural screening. The screened calli derived clones were the first to take infection although plantlets from both the sources took infection and wilted completely. Infection and wilting was not influenced by the age of the calli-clones.

30. The leakage of electrolytes from leaves induced by culture filtrate of *P. capsici* was more in screened calliclones as compared to unscreened calliclones.

31. The clones from screened calli registered higher lesion development as compared to the clones from unscreened calli, when inoculated with culture disc of *P. capsici*.

32. The cultivars differed significantly in the intensity of lesion development. The lesion diameter score recorded for Cheriakanyakkadan was 14.68 per cent lesser than that recorded for Kalluvally.

33. Comparative efficacy of the three callus screening methods were studied in the cultivar Kalluvally. Clones screened by method 3 produced more tolerant plants even though the overall LDS was more in the clones derived from the method.

34. Highly significant somaclonal variation in electrolyte leakage was observed in calliclones of the cultivar Kalluvally while no significant variation was observed in other cultivars. High amount of somaclonal variation in lesion development was also observed in the cultivar Kalluvally as compared to Cheriakanyakkadan. It was found that longer the period in culture, higher was the variation observed in lesion development.

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* Originals not seen

Appendices

APPENDIX-I

Chemical composition of different media used in the study

Constituents	Quantity (mg/l)
1	2
(1) Chemical composition of MS (1962) medium	
Major elements	
$CaCl_2.2H_2O$	440.000
$FeSO_4.H_2O$	27.800
KNO_3	1900.000
KH_2PO_4	170.000
$MgSO_4.7H_2O$	370.000
$NH_4 NO_3$	1650.000
Na_2.EDTA	37.300
Minor elements	
$CaCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.025
H_3BO_3	6.200
KI	0.830
$MnSO_4$	22.300
$Na_2MoO_4.2H_2O$	0.250
$ZnSO_4$	8.600
Organic constituents	
Glycene	2.000
Myoinositol	100.000
Nicotinic acid	0.500

Contd.

Appendix-I. Continued

1	2
Pyridoxine HCl	0.500
Thiamine HCl	0.100
Sucrose	30.000 g
Agar	7.500 g
(2) Chemical composition of Ribeiro (1978) medium	
Major constituents	Quantity/l
Glucose	4.5 g
L-asparagine	0.1 g
KNO_3	0.15 g
KH_2PO_4	1.0 g
$MgSO_4.7H_2O$	0.5 g
$CaCl_2$	0.1 g
ß sitosterol	30 mg
Microelements	Quantity/100 ml
$Na_2MoO_4.2H_2O$	41.1 mg
$ZnSO_4.7H_2O$	87.8 mg
$CuSO_4.5H_2O$	7.85 mg
$MnSO_4.H_2O$	15.4 mg
$Na_2B_4O_7$	0.5 mg
$FeCl_3.6H_2O$	50 mg
Organic constituents	Quantity/l
Agar	14 g

Contd.

Appendix-I. Continued

1	2
Thiamine	1.0 mg
(3) Composition of Potato Dextrose Agar medium	
Potato	200 g
Agar	20 g
Distilled water	1 l
Dextrose	20 g
(4) Composition of Carrot Agar Medium	
Carrot	200 g
Agar	18 g
Distilled water	1 l

APPENDIX-II

Month wise planting out, number of plants wilted during hardening, number of plants wilted after hardening and final survival of plantlets

Month	Total planted out	Mortality during hardening	Survival	Mortality after hardening	Final survival
October 1993	2	0	2	0	2
December 1993	34	17	17	0	17
January 1994	22	15	7	2	5
February 1994	36	21	15	1	14
March 1994	18	9	9	2	7
April 1994	84	36	48	6	42
May 1994	138	83	55	6	49
June 1994	103	46	57	8	49
July 1994	339	92	247	40	207
August 1994	237	99	138	45	93
September 1994	289	95	194	40	154
Total	1302	513	789	150	639

APPENDIX-III

Monthly weather data recorded during the period from 1992-1994 at College of Horticulture, Vellanikkara

Parameters	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
Year 1992												
Max. t	32.6	34.5	36.9	36.3	33.8	30.1	28.8	28.9	30.1	30.7	31.0	31.1
Min. t	20.9	21.8	22.8	24.4	24.8	23.7	22.7	23.3	23.1	22.9	23.1	22.3
Rain (mm)	0.0	0.0	0.0	48.6	90.6	979.8	874.5	562.9	302.9	386.7	376.7	2.0
Rainy days	0	0	0	3	6	22	26	25	17	14	12	0
Mean RH (%)	53	65	61	65	73	84	87	88	82	82	77	61
Sunshine (hours)	9.0	9.2	9.2	8.8	7.4	3.3	2.1	2.7	4.1	4.6	5.3	8.9
Year 1993												
Max. t	32.6	34.1	35.4	34.5	34.4	30.1	28.5	29.6	30.6	30.7	31.5	31.6
Min. t	20.7	22.0	23.7	25.0	24.8	23.9	22.9	23.4	23.1	23.4	23.6	23.1
Rain (mm)	0.0	6.6	0.0	32.1	131.1	700.3	661.6	276.7	85.3	519.0	74.6	18.0
Rainy days	0	2	0	2	6	22	29	20	9	16	4	2
Mean RH(%)	53	62	63	69	74	86	87	87	81	83	73	66
Sunshine (hours)	8.1	9.4	9.0	9.1	6.5	3.3	2.4	4.8	6.4	4.8	5.8	7.5
Year 1994												
Max. t	32.9	34.8	36.2	34.9	33.6	28.9	28.6	30.0	31.8	32.3	31.8	32.2
Min. t	22.6	23.1	23.7	24.4	24.7	22.9	22.4	22.8	23.2	22.7	23.3	22.2
Rain (mm)	19.4	1.7	21.0	165.2	124.2	955.1	1002.1	509.2	240.5	358.2	125.3	0
Rainy days	1	0	1	10	7	27	29	20	8	20	5	0
Mean RH(%)	58	59	59	74	75	90	91	85	78	80	68	58
Sunshine (hours)	9.1	8.7	9.3	8.0	8.0	2.1	1.4	3.0	7.3	6.7	8.1	10.6

APPENDIX-IV

Correlation coefficients worked out with weather parameters and mortality and final survival of black pepper plantlets

Variables	Weather parameters					
	Maximum temperature	Minimum temperature	Rainfall	No. of rainy days	Relative humidity	Sunshine hours
Mortality during hardening	-0.438	-0.069	+0.419	+0.496	+0.598*	-0.471
Survival	-0.552	-0.351	+0.573	+0.597*	+0.634*	-0.582*
Mortality after hardening	-0.534	-0.394	+0.491	+0.547	+0.593*	0.593*
Final survival	-0.553	-0.324	+0.588*	+0.605*	+0.642*	-0.579*

APPENDIX-V

Cultivar wise details of the calli clones derived from screened and unscreened calluses

Sl. No.	Cultivar	Treatments	No. planted out	No. wilted during hardening	Survival after hardening	No. wilted after hardening	Final survival
1	Karimunda	Screened (Method I)	21	14	7	1	6
		Unscreened	59	23	36	4	32
2	Balankotta	Screened (Method I)	41	20	21	7	14
		Unscreened	46	24	22	6	16
3	Cheriakani-kkadan	Screened (Method I)	63	21	42	5	37
		Unscreened	145	44	101	10	91
4	Culture No.1	Screened (Method I)	227	96	131	29	102
		Unscreened	82	25	57	10	47
5	Kalluvally	Screened (Method I)	98	42	56	12	44
		Screened (Method II)	32	17	15	3	12
		Screened (Method III)	227	101	176	36	140
		Unscreened	185	77	108	22	86
6	Panniyur-1	Unscreened	26	9	17	5	12

APPENDIX-VI

Abstract of analysis of variance for the effect of different treatments

Sl. No.	Table No.	Character	Treatment DF	Treatment MSS	Error DF	Error MS	CD (0.05)
1	2	3	4	5	6	7	8
1	1	Influence of incubation period and type of culture on the accumulation of toxic metabolite(s)	2	1.401	9	0.003	0.088
2	2	Effect of autoclaving of CCF on symptom development	5	1.130	18	0.003	0.081
3	3	Effect of dilution of CCF on symptom development	3	0.829	12	0.002	0.069
4	6a	Electrolyte leakage from leaves of pepper genotypes	5	10903.12	12	5.500	4.172
5	6b	Electrolyte leakage from calli of pepper genotypes	5	2716.36	12	0.889	1.677
6	6c	Electrolyte leakage from calli of *in vitro* seedling explants	5	4956.10	10	0.733	1.562
7	7	Effect of dilution of CCF on electrolyte leakage from calli					
		Concentration	3	696.47	20	0.600	0.722
		Varieties	4	354.91	20	0.600	0.807
		Variety x concentration	12	187.613	20	0.600	1.615
8	8a	Effect of CCF on callus necrosis					
		Varieties	4	0.154	19	0.002	0.044
		Concentration	3	0.173	19	0.002	0.039
		Variety x concentration	12	0.004	19	0.002	0.087

Contd.

Appendix-VI. Continued

1	2	3	4	5	6	7	8
9	8b	Effect of CCF on callus necrosis					
		Varieties	4	0.175	19	0.005	0.070
		Concentration	3	0.144	19	0.005	0.063
		Variety x concentration	12	0.003	19	0.005	1.409
10	18	Effect of callus screening (Method 2) on callus necrosis					
		Treatment	7	1.359	39	-	0.075
		Variety	4	0.277	39	-	0.059
		Interaction	28	0.012	39	-	0.167
11	22	Effect of callus screening (Method 3) on callus necrosis					
		Varieties	4	0.129	9	0.008	0.174
		Treatment	1	0.949			0.110
		Variety x treatment	4	0.119			0.249
12	27	Effect of gamma irradiation on callusing and callus growth					
		Varieties	3	0.286	23	0.016	0.106
		Dose	5	0.104	23	0.016	0.234
		Variety x dose	15	0.052	23	0.016	0.260
13	31a	Response of black pepper cultivars to callusing					
		% of callusing					
		Varieties	5	0.077	12	0.009	0.169
		No. of days taken					
		Varieties	5	0.068	12	0.014	0.211

Appendix-VI. Continued

1	2	3	4	5	6	7	8
14	31b	Response of black pepper cultivars to callusing					
		Callusing (%)					
		Varieties	4	0.097	10	0.008	0.163
		Days taken for callusing					
		Varieties	4	0.246	10	0.045	0.386
15	31c	Response of black pepper cultivars to callusing					
		Days taken for callusing					
		Varieties	4	0.003	10	0.001	0.058
16	31d	Effect of source of explants on callusing					
		% of callusing					
		Explant	2	0.193	12	0.020	0.195
		Days taken for callusing	2	0.454	12	0.073	0.373
17	31e	Effect of cultivars and source of explants in days taken for shoot induction and % regeneration					
		No. of days taken					
		Varieties	4	2.908	25	0.031	0.209
		% of regeneration					
		Varieties	4	0.117	25	0.008	0.106
18	33	Effect of containers in the proliferation of shoots					
		Containers	4	4.112	55	0.061	0.203

Contd.

Appendix-VI. Continued

1	2	3	4	5	6	7	8
19	34	Response of different cultivars to shoot proliferation					
		Varieties	4	10.369	55	0.069	0.216
20	35	Response of cultivars to the recovery of rootable shoots					
		4th S.C Variety	3	0.450	44	0.084	0.251
		5th S.C Variety	4	5.921	55	0.079	0.230
		6th S.C Variety	4	7.066	55	0.050	0.184
21	36	Response of cultivars to rooting and root growth					
		Days for root initials to appear					
		Variety	4	0.147	55	0.011	0.086
		No. of roots					
		Variety	4	2.196	55	0.209	0.376
		Root length					
		Variety	4	1.164	55	0.437	0.543
		Root thickness					
		Variety	4	0.218	55	0.034	0.151
22	41	Effect of age of the calliclones plants on the no. of plants wilted					
		Age	2	0.014	22	0.019	

ABSTRACT

Investigations on the exploitation of somaclonal variation for screening for resistance/tolerance to *Phytophthora* foot rot disease in black pepper were carried out at the Plant Tissue Culture Laboratory of the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara during September 1991 to January 1995.

Calliclones of different black pepper cultivars viz. Kalluvally, Cheriakanyakkadan, Balankotta, Karimunda and Panniyur-1 were produced with and without applying *in vitro* selection pressure using toxic metabolite(s) of *Phytophthora capsici*. *In vitro* induction of mutation using gamma irradiation and partial purification of the toxic metabolite(s) present in the culture filtrate of *P. capsici* were also attempted in the present study.

Leaf puncture bioassay of the concentrated culture filtrate (CCF) of *P. capsici* showed that toxic metabolite(s) were accumulated in the culture filtrate. The symptoms produced by CCF were quite typical of natural and artificial infection by *P. capsici*. Concentrated culture filtrate induced quick electrolyte leakage from leaves and calluses.

Concentrated culture filtrate induced necrosis on susceptible calli. The cultivars showed significant variation in callus necrosis. Prolonged duration of selection/screening with CCF totally inhibited the regeneration potential of the calli. Concentrated culture filtrate was not found to inhibit shoot proliferation and shoot growth in already regenerated cultures but inhibited the root growth.

In the three direct selection/screening methods tried for calli viz. growing in CCF incorporated MS medium (Method 1) shaking in CCF incorporated liquid MS medium (Method 2) and double layer culture technique (Method 3), cultivars showed significant differences in callus necrosis and callus growth. Direct screening of calli was not found to inhibit the regeneration of shoots, shoot proliferation and recovery of rootable shoots but affected the root growth adversely.

Gamma irradiation of calli using ^{60}Co source did not give any better response to *in vitro* screening.

The toxic metabolite(s) present in the culture filtrate could not be separated by organic solvent fractionation. However ion exchangers like Dowex 1 and Dowex 50 could be used for separation of the toxic fraction from the filtrate.

The response of five different cultivars at various stages of development of cultures when compared, it was found that the cultivars differed significantly in callusing, callus growth, regeneration of shoots, recovery of rootable shoots and root growth.

The clones regenerated from screened and unscreened calli were further tested for resistance/tolerance to *P. capsici* using different methods of screening viz. natural screening (keeping in infected field), screening by electrolyte leakage method and screening by artificial inoculation of culture disc of *P. capsici*. None of the regenerated calliclones were found to be resistant to the disease in natural screening. When the tolerance level of the regenerated calliclones was looked into, the performance of the unscreened calli derived clones was found better as compared to

the screened calli derived ones. The calliclones of different cultivars differed significantly in the tolerance/susceptibility reaction to the disease. The calliclones of Cheriakanyakkadan recorded greater degree of tolerance to the disease when compared to others.

Among the cultivars studied, Kalluvally exhibited high rate of somaclonal variation.