CHAPTER IV

DISCUSSION

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This research endeavour had four directions of special emphasi : First, identify the optimal cultural conditions for plantlet regeneration from various explants of C. cajan of different genotypes (T-15-15, Bandapalera, NP(WR)15, BDN2, GAUT-82-90); second, to attemp in vitro embryo rescue and ovule culture methods for obtaining intervarietal and intergeneric viable hybrid embryos and plants; third, varietal screening of C. cajan for wilt disease, using culture filtrate of F. udum at the whole plant and leaf disc level; and four, to obtain a resistant line by applying selection pressure (culture filtrate of F. udum).

(A) MORPHOGENETIC RESPONSE FROM SEEDLING EXPLANTS OF CAJANUS CAJAN (L.) MILLSP.

Biotechnology, which comprises of a number of novel techniques, such as somatic hybridization, somaclonal variant selection and genetic transformation of cells, has a great potential in supplementing the efficiency of the conventional methods for crop improvement. This potentiality can only be realized by developing protocols for sustained and reproducible regeneration of plants from cultured cells. The tissue culture methods of crop improvement involve manipulation and selection at cellular level. Again such manipulations are effective only when whole

plants can be recovered with high precision and in sufficiently large number. This is necessary to permit the study of their properties and performances.

Success in regeneration of plants from organ, tissue and cell levels depends on the understanding of organogenesis and/or embryogenesis as processes and their precise experimental regulation. Many kinds of differentiated cells in the plant are flexible to differentiate and redifferentiate (Gautheret 1985) under appropriate stimuli that are provided during culture process. The degree of regression a cell can undergo would depend on the cytological and physiological states it has reached *in situ* (Gautheret 1966). The earlier studies on elucidating mechanisms underlying organogenesis had a land mark in Skoog and Millier's (1957) classical finding of quantitative interaction of auxin and cytokinin as the basic regulatory mechanism. Higher levels of auxin to cytokinin favoured rooting from tobacco pith tissue and the reverse shoot induction. This regulatory mechanism holds good for a number of systems though there are some exceptions like *Medicago* wherein exactly the reverse situation operated. High auxin cytokinin ratio was reported to have given rise to

shoots and the low to roots (Walker et al 1978). Steward and his colleagues developed an in vitro system with carrot tissues which required only the removal of the auxin 2,4-D from cell cultures to elicit adventive embryogenesis (Steward 1958). Although mechanism of hormonal balance has not been universally demonstrated (Halperin 1973), these two distinct techniques still remain as the major alternative means used to obtain the de novo organization of plant metistems from cell cultures in vitro. However, many other hormonal. nutritional, physical, spatial and temporal factors are involved in eliciting a particular morphogenetic response.

Induction of callus has been by far a comparatively easier operation with crop legume systems, but it has still been difficult to regenerate plants from them along the organogenetic or embryogenetic pathways. A large number of workers have reported induction and establishment of callus cultures from almost all the economically important leguminous plants by manipulating various factors. Most of these works have not led to plant regeneration due to their inherent resistance to it. Thus, the earlier studies dealt with callus induction

(Mehta 1966), physiological aspects of growth (Mehta et al 1967) and morphogenesis (Mehta 1980) and respiration (De Klerk Kiebert 1983) or the lines of work that would find application on development of regeneration protocols like cell line isolations applying selection pressures (Huges 1978, Pandey and Ganapathy 1984).

Cajanus cajan was used in our laboratory for growth response of callus (Sreedhar 1986) using B_5 (1968) medium. Our later studies showed better responses of callus with modified Murashige and Skoog (MMS) medium, i.e. salts of MS (1962) and vitamins of B_5 (1968) medium, and hence MMS medium was used in all the subsequent experiments, except the experiments on testing the influence of nutrient formulations.

The results obtained with various explants of pigeonpea var. Bandapalera are presented in Chapter III, section A. It is, very clear that for initial establishment of culture from the seedling explants tested i.e. epicotyl, leaf and cotyledon, the later gave best response on MMS medium supplemented with only cytokinins (BAP as well as KN) and adenine sulphate. No auxin was found necessary for

this purpose. However regeneration was not obtained on this nutrient medium. The medium containing auxin and/or BAP alone as cytokinin proved ineffective for induction. Though callusing was noticed on all the nutrient media, the cultures turned brown after two weeks, except on medium containing BAP, KN and AS where the callus remained green and compact for 3 weeks. On prolong incubation on the same medium, the callus grew further and became nodulated with bud primordia. However, their further development was inhibited on the same medium. The best morphogenetic response was registered when these explants were transferred, after removing the primary callus to the medium containing low level of auxin (IBA 0.1 mg/l) and gibberelin (GA_3 0.05 mg/l) in case of epicotyl and cotyledonary explants. In case of leaf explants, TIBA (0.5 mg/l) was found to be more effective than IBA but with low level of kinetin.

Simmilar results are reported by Sreedhar (1986) in C. cajan, var. T-15-15 on B_5 medium containing the same levels on cytokinins as used in the present investigation but with one tenth of the adenine sulfate concentration. The best regeneration medium

reported by Sreedhar (1986) was hormone free. In contrast to these reports, Suresh Kumar et al (1983, 1984) demonstrated direct shoot formation on Blayde's medium from leaf, epicotyl, shoot apices, root as well as cotyledonary explants of C. cajan. However, they also reported callus induction on one medium and regeneration from callus on the other.

Behaviour to Cajanus explants in the present investigation markedly differ from that of some earlier reports (Suresh Kumar et al 1983, 1984, Mehta and Mohan Ram 1980) where induction medium itself was also the regeneration medium as both the processes occurred on the same medium. In the present investigation, On the other hand, there is a clear distinction and temporal separation of the two phases, namely induction and regeneration.

It is evident from the studies that response of the explants was directly dependent on the hormonal composition of the induction medium. The suppression of differentiation by auxin in the induction medium was clearly noticed in case of all the explants, as evident by poor response of these explants on regeneration medium. Such suppressive effect of auxin on differentiation is not without precedence. Ramawat

et al (1977) in Crotolaria, Meijer (1982) in Stylosanthes humilis and Sreedhar (1986) in Cajanus cajan var. T-15-15 have reported similar inhibiton by auxin.

In general, auxin inhibit bud formation (Paterson 1975) although there are reports that low auxin concentrations stimulated shoot regeneration in systems where high concentrations were inhibitory (Bonnett and Torrey 1965, Kefford and Caso 1972). In the present studies, in case of leaf explants, shoot differentiation was completely inhibited by even low concentration of auxin (0.1 mg/l IBA) and incorporation of TIBA was found beneficial for shoot bud induction alongwith Kinetin (regeneration medium B). This was not the case with epicotyl and cotyledonary explants where low auxin concentration stimulated shoot buds/embryos formation (regeneration medium A). Thus, it appears that an assessment with respect to auxin must be made for the specific case in question. More fundamental studies are required to pinpoint how exactly exogenous auxin suppressed/ stimulated the process and whether the endogenous auxins are active in inducing differentiation in these cases.

Adenine sulfate is known to counteract auxin imposed apical dominance by developing vascular connections in the buds (Thimann and Sachs 1966). Incorporation of adenine sulfate in the induction medium enhanced the response from 50 % to over 80% for shoot differentiation and could partialy reverse the auxin induced suppression of shoot differentiation in C. cajan (Sreedhar 1986). Such quantitative counter-action of a cytokinin to auxin suppressive effect is known (Skoog 1971). The beneficiary effect of incoproration of adenine sulfate in induction medium is also evident in the present investigation both for shoot differentiation from epicotyl and leaf explants as well as for embryo induction from cotyledonary explants.

Mehta and Mohan Ram (1980) failed to regenerate shoots from epicotyl explants of "Prabhat" variety of Cajanus cajan. On the other hand, Sreedhar (1986) reported successful regeneration of shoots from the epicotyle explants of the same variety and concluded that the failure of Mehta and Mohan Ram (1980) was because of (i) presence of auxin (NAA at 10^{-6}M) in the induction medium and (ii) no sequential transfer of explants form induction to regeneration medium.

Both these factors are found essential also for the variety Bandapalera for successful shoot induction as was reported by Sreedhar (1986) for variety T-15-15.

GA_3 induced shoot formation was reported in Atriplex by Wochok and Sluis (1980) and in Cajanus cajan by Suresh Kumar et al (1983). Incoportion of GA_3 at 0.05 mg/l was also found inducive for shoot differentiation as well as for rooting of shoots. The regenerated shoots, originating either from epicotyl or leaf explants, were successfully rooted on MMS medium having half the concentration of all the nutrients and supplemented with 0.1 mg/l IBA and 0.05 mg/l GA_3.

These informations generated in the present investigation can be used to effectively promote the regeneration of pigeonpea plantlets from seedling explants of var. Bandapalera via callus formation. Since callus tissues are a source of genetic diversity, regeneration from such cells might help in augmenting the existing gene pool for the genetic improvement of this crop.

(B) SOMATIC EMBRYOGENESIS FROM COTYLEDONS

One of the objectives of the present investigation was to attempt *in vitro* approaches to develop wilt resistance in *Cajanus cajan*. An essential prerequisite for use of *in vitro* approaches in crop improvement is a high regenerative system. Earlier, plant regeneration from different explants of *Cajanus cajan* seedlings has been reported via organogenesis (Mehta and Mohan Ram 1980, Sureshkummar et al 1983, Sreedhar 1986, Anonymous 1987), but the frequency of regeneration was low. The frequency of regeneration through direct organogenesis from seedling explants attained in the present investigation is quite high as described and discussed earlier.

Recently, plant regeneration via *in vitro* embryogenesis in *C. cajan* has been reported by few workers (Anonymous 1987, Vaidyanath and Amrith Sagar 1990). These workers have used, embryogenic materials, i.e. mature and immature embryos. Earlier, Kanta and Padmanabhan (1964) had reported plant regeneration from embryo segments of *Cajanus cajan*. In the present investigation a procedure for the regeneration of whole plants of pigeonpea through somatic

embryogenesis from non-embryogenic material, i.e. distal half of cotyledon is standardized in var. Bandapalera of Cajanus cajan.

In most of the reports soaking in water prior to inoculation on the nutrient medium for somatic embryogenesis was found necessary for the separation of immature/mature embryos from cotyledons/seeds. Anonymous (1987) soaked the seeds of pigeonpea genotypes for three hours in sterile distilled water for the same purpose. In the present studies, however, soaking of seeds on a gyratory shaker for 16 to 20 hrs not only stimulated the separation of non-embryogenic tissues from embryogenic material but also ehnaced embryo induction frequency from the excised cotyledons. The presence of phenolic compounds adversely affecting morphogenetic response of cotyledons was visualized by Mehta and Mohan Ram (1980), Suresh Kumar et al (1983) and Sreedhar (1986). It is likely that prolong soaking of seeds as in the present investigation resulted into leaching of phenolic compounds which in turn, was responsible for enhanced response of the excised cotyledons. However, this needs to be verified by analysis of the leachate for the presence of inhibitory phenolic compounds, if any.

The process of embryogenesis is highly dependent on the growth regulators. Attention is being focused on the type, concentration and time of application of plant growth regulators. Of the auxins tested, 2,4-D has been found most effective and in most of the plant species gradual removal of 2,4-D in the culture medium has been reported for the formation of somatic embryos (Rangaswamy 1986). On the other hand, Lazzeri et al (1987) demonstrated that in soybean, somatic embryos induced by NAA appeared more like zygotic embryos than those induced by 2,4-D. Importance of exogenous auxin (2,4-D and NAA) in regulating somatic embryogenesis in Cajanus cajan was also showed by Vaidyanath and Amrith Sagar (1990). However, in the present studies, maximum response, in terms of per cent responsive cotyledons as well as in frequency of embryo induction was registered with cytokinin alone (5.0 mg/l BAP and 0.5 mg/l Kn). Incorporation of auxin (NAA) from 0.5 to 20 mg/l along with BAP in the induction medium suppressed the embryogenic response.

Cytokinins do play an important role in the induction, especially for maturation and germination of somatic embryos (Fujimura and komamine 1980). Data on the nature and amounts of cytokinins used

for induction of somatic embryogenesis has been compiled by Evans et al (1981). Cytokinins have been implicated as a necessary factor for somatic embryogenesis in some other species (Mullins and Srinivasan 1976, Sundahl and Sharp 1977, Kavathekar and Johri 1978, Desai et al 1986, Maheswaran and Williams 1986, Bauchan 1987, George and Eapen 1988 and Liwang et al 1990). In the present investigation in C. cajan, was also the optimum concentrations required to support highest embryogenic response, both on the basis of per cent responsive cotyledons and frequency of embryo induction was 5.0 mg/l BAP + 0.5 mg/l Kn with 50 mg/l Ads.

These results are not without precedence. Anonymous (19870 reported that all the auxins tested either alone or in combination with cytokinins failed to induce somatic embryos either from explants or from callus of three different pigeonpea genotypes. The auxin promoting effect on somatic embryogenesis of Vidyanath and Amrith Sagar (1990) and auxin suppressive effect of Anonymous (1987) and in present studies with Cajanus cajan may be attributed to the different genotypes and the explant sources used besides different nutrient formulations. Vaidyanath

and Amrith Sagar (1990) had used embryogenic material asexplant source and L_6 as nutrient medium as opposed to MMS nutrient formulation used by Anonymous (1987) and in the present studies. Similarly, Anonymous (1987) used embryogenic material as explant source as against non-embryogenic material in the present studies.

Thee are reports of the influence of embryo size (Tetu et al 1990) and embryo age (Chen et al 1990) affecting somatic embryogenesis in legumes. In the present studies for the first time size and age of the cotyledon, from which embryonic axis was removed, influencing somatic embryogenesis is demonstrated. Increase in response in terms of per cent responsive cotyledons and frequency of embryo induction was registered with increasing age and size of the cotyledons in case of pigeonpea, Var. T-15-15. All the cotyledons were responsive either forming callus or embryos, if their size was 7 mm x 9 mm and age 35 days after anthesis. Frequency of embryo induction was also very high in these cotyledos. This is in contrast with the observation made by Chen et al (1990) with inter specific hybrid embryos of Vigna glabrescens and V. radiata where

cotyledons derived from young embryos were more responsive than those derived from older embryos.

Nitrogen source, especially reduced N in the medium, is another important factor affecting embryo formation from somatic cells. The role of nitrogen compounds in embryogenesis has been studied extensively. Tazawa and Reinert (1969) had observed that embryogenesis in vitro could be induced by both inorganic and organic compounds. When either NH_4^+ or NO_3^- was sole source of nitrogen in the culture medium, somatic embryogenesis did not occur or incidence was infrequent. When the ratio of NO_3^- to NH_4^+ was 2 : 1, as in MS medium, somatic embryogenesis is reported to occur with the highest frequency in egg plant (Gleddie et al 1983).

In many systems it is found that casein hydrolysate (CH0 and amino acids stimulate somatic embryogenesis (Stuart and Strickland 1984, Stuart 1985, Mauro et al 1986, Swedlund and Locy (1980. For instance, 1 or 2 g/l CH or 4.4 mM glutamine with 3.1 mM proline caused 20-30% increase in the number of embryoids in Medicago sativa (Meiger and Brown 1988).

The effect of amino acids on somatic embryogenesis has been well demonstrated in several different species (Ronchi et al 1984, Armstrong and Green 1985, Duncan et al 1985). Among the amino acids used as sole sources of nitrogen, glutamine or its products is critical for embryogenesis in carrot (Wetherell and Dougall 1976, kamada and Harada 1979b, Sharp et al 1980). Glutamine mose readily promoted embryogenesis in carrot tissue culture (kamada and Harada 1979b). Glutamine has also proved to be the most effective amino acid for the growth of excised embryo (Paris et al 1953, Rijven 1955, Matsubara 1964, Mannier 1978, Tetu et al 1990). Aspargine too was found effective in enchancing embryo growth, but at the same time it was found inhibitory in some of the species (Tetu et al 1990).

Since glutamine (Meiger and Brown 1988) and aspargine (Tetu et al 1990) have been found most effective of amino acids for embryo maturation and/or germination, their effect on the induced embryo of C. cajan was examined in the present study. However, neither of them, at the concentration (20 mg/l) tested, singly and in combination, proved promotory,

i.e. they reduced embryo maturation and germination into complete plants from the already induced embryos.

Amino acid analogue such as 5-M-T is found to evoke positive response for embryo maturation in a few cases (Desai et al 1986 and Urinikrishnan et al 1990). They have successfully used a tryptophan analogue, 5 methyl tryptophan to prevent recallusing and precocious germination of somatic embryos in soapnut. However, in inflorescence axis cell suspensions of Plantago ovata, 5-M-T proved ineffective to induce normal somatic embryogenesis and to prevent prococious germination of embryos (Barot 1991).

Abscisic acid too is shown to play a special role in somatic embryo maturation and germination in many cases, particularly in the Umbeliferous, caraway and carrot (Ammirato 1983). ABA also benefited somatic embryo maturation in soybean (Ranch et al 1985, Ghazi et al 1986) and sunflower (McCann et al. 1988). In pennisetum Purpureum, an inhibitor of ABA synthesis, inhibited embryogenesis (Rajasekaran et al. 1987).

Timely addition of ABA to embryogeneic culture increased the number of heart-shaped embryos in soybean (Phillips and Collins 1981). The frequently observed absence of heart shaped somatic embryo in cultures of several dicotyledonous species points to a stage-development role of naturally occuring growth inhibitors such as ABA in normal embryogenesis. Moreover, continued presence of ABA is inhibitory for somatic embryos to develop into plantlets in Trachyspermum amini (Barot 1991).

In the present studies, ABA (0.025-0.25 mg/l) when used alone or in combination wih amino acids (glutamine and asparagine) did not improve the situation.

Ethylene is known to inhibit somatic embryogenesis (Ammirato 1983). Addition of ethylene antagonists norbornadiene and silver nitrate increased plant growth in maize cultures (Songstad et al 1988). Two ethylene antagonists, namely silver nitrate and salicylic acid, were therefore tried in the present investigation to increase emrbyo maturation and germination. Though salicylic acid (100-200 mg/l) had little effect, silver nitrate (20 mg/l) enhanced somatic embryo maturation and germination (75-80%).

Meijer et al (1988) had also reported inhibition of somatic embryogenesis by salicylic acid at concentration which do not inhibit ethyelene biosynthesis and growth. In the present investigation, the low concentration of salicylic acid used may not be sufficient to inhibit ethylene biosynthesis and, on the other hand, high concentration may have affected growth adversely.

Somatic embryogenesis reported in pigeonpea (Anonymous 1987, Vaidyanath and Amrith Sagar 1990) had not emphasized differences in response due to the genotypes. However, genotypic effect on somatic embryogenesis has been reported for certain species (Tomes 1985, Brown 1988) and further, they have also demonstrated somatic embryogenesis in all the genotypes by manipulation of exogeneous factors such as culture media. In the present studies four genotypes, viz. T-15-15, GAUT-82[illegible]-90, Bandapalera and NP (WR)15, and six salt formulations were tested to find genotypic difference as well as effect of salt formulations on embryo induction. Of these four genotypes, two viz. T-15-15 and GAUT-82[illegible]-90 are susceptible for wilt and the other two genotypes, viz. Bandapalera and NP (WR) 15 are tolerant to the

said pathogen. The variation in the response due to genotypes was clearly evident in the present studies. Similarly, manipulation of culture medium composition also medified the degree of response in terms of the frequency of embryo induction and per cent response of cotyledons. However, healthy embryos capable of producing green leafy shoots were obtained only on MMS medium in all the varieties. Though single salt formulation, i.e. MMS, emerged as the most suitable for all the genotypes examined here for obtaining plants through somatic embryogenesis, results have clearly indicated that it may be possible to increase the response of a particular genotypic by changing the level of components in the culture medium.

In the present investigation, the protocol for obtaining plants through somatic embryogenesis with high frequency is attained. The protocol described provided a reliable system for the regeneratiion of plants from somatic tissue of pigeonpea and is applicable to a range of genotypes. While the efficiency of generation is yet lower than that available for crop species of the Solanaceae and Cruciferae, the observation that a single isolated distal halt of cotyledon can directly produce 40 to 90 and more somatic embryos, augurs well for its application in pigeonpea improvemment programme.

(C) FIELD STUDIES

C.1 Plantlet regeneration from immature embryos

The cultivated pigeonpea, Cajanus cajan has many related wild species in the genus Atylosia, some of which have desirable characters such as disease and pest resistance (Remanandam 1980, Reddy et al 1980). In order to transfer disease resistance trait to cultivated pigeonpea, crosses were attempted between A. lineata and C. cajan var. T-15-15 and var. GAUT 82-90 in the present investigation. These crosses were however unsuccessful. Though two pods were obtained in the cross between A. lineata with Var. T-15-15 seed setting was not noticed in the pods. Similar failure in crosses between Atylosia and pigeonpea were reported earlier by a number of workers (Pundir 1981, Dhanju et al 1985). The crossing barriers were also examined by Pundir and Singh (1985) and they found that inhibition of pollen tube growth was the barrier to crossability. Dhanju et al (1985) and Sateeshkumar et al (1985) reported beneficial effect of growth hormones in the crosses between pigeonpea and Atylosia. No such treatments were provided in the present investigation. Probably,

the temperature which was considerably high during the period crosses were made (the flowers of A. lineata were available only during May) had adverse influence on the crosses.

Alternatively, the possibility of transferring wilt tolerance character from the tolerant varieties of pigeonpea, i.e. NP (WR)15 and Bandapalera (used as male parents) to the susceptible but high yielding varieties, T-15-15 and GAUT 82-90 (used as female parents) was attempted. The hybridization success ranged from 3.3 to 5.2% depending upon the parental genotypes. Hybrid embryos were reared to complete plantlets in all the crosses by in vitro embryo rescue techniques.

The technique of embryo culture was standardized in the present investigation using selfed T-15-15 embryo as a model system. Incorporation of 2,4-D or NAA resulted in more callus development and low frequency of plantlet regeneration as compared to IAA as the auxin source. Monnier (1978) had suggested that hormones should not be added to the embryo culture media because they bring about structural abnormalities.

He felt that embryos are autonomous for most of the growth regulators. However, as evident from the data that recovery of plants was low when growth regulators were not added to the Bladey's culture medium, Raghavan and Torrey (1964) reported the necessity of IAA, Kinetin and adenine sulfate with 2% sucrose for successful embryo culture of Capsella. Though growth regulator supplement was not necessary for the embryo growth in the present studies, addition of IAA, kinetin and coconut water with 2% sucrose enhanced plant recovery considerably. The same medium was utilized for successful plant recovery from eight different genotypes of pigeonpea, although genotypic differences were quite evident regarding per cent regeneration of plants.

C.2 *In vitro* induction of germination in ovules

The difficulty of growing very young or minute embryos prompted us to culture ovules. Culturing of ovules is advantageous as they can be excised even at the zygote stage and are though to provide a "maternal environment" for the developing embryo. Most of the Cajanus-Atylosia crosses are successful only when Cajanus is used as the female parent, hence the selfed

ovules from Cajanus were used for standardizing the techniques.

It was evident from the data obtained (Table D-4) that Kn, IAA/NAA, CH and GA_3 not only supported growth of Cajanus ovules but also favoured vigorous callusing from which eventually plantlets were recovered. Similar observations were made earlier by Bimal (1988) in tobacco and Cohen et al (1984) in Lens. It has also been reported that growth supplements such as kinetin (Maheshwari and Lal 1961, Moss et al 1988), Casein hydrolysate (Bajaj 1964) and GA_3 (Moss and Stalver 1987) favoured the growth of ovules. The successful growth and germination of ovules in vitro was also found to be related to the age of the ovules in culture. Ovule age affecting the response was also reported in Nicotiana species (Siddiqi 1964) and Arachis hypogaea (Moss et al 1988, Moss and Stalker 1987). Wakizara and Nakujima (1974) reported beneficial effect of increasing sucrose level for ovule germination in Petunia hybrida. However, in the present studies sucrose had very little effect on ovule germination. Similar observation was made by Cohen (1984) in Lens.

Though on most of the media tested in the present investigation, majority of the ovules failed to show further development and germination, 20-25% germination of ovules was obtained on Blayed's basal medium with 2-8% sucrose or supplemented with kinetin, CH and GA_3 and on MMS medium supplemented with IAA/NAA, kinetin and CH. These germinated ovules developed either single shoot or multiple shoots after 5-6 weeks of incubation period . The results obtained in the present studies might help in future with further refinement in the technique in obtaining high frequency of Cajanus-Atylosia hybrids. Sequential culture of ovary, ovule and embryo has been very effectively demonstrated by Agnihotri (1992) for overcoming post-fertilization barriers in crosses between Brassicas and their wide allies. Such an approach too needs to be explored with Cajanus-Atylosia crosses.

(D) PATHOLOGICAL STUDIES

In the conventional "Sick plot" method for screening pigeonpea plants for wilt resistance, according to Nene et al (1981), a well isolated plot of desirable size is selected and inoculated with

chopped stubbles of wilted plants from other fields and watered well. The varieties to be screened then sown in this "infected" plot, ensuring good plant population and carrying out normal agronomic operations. Preliminary results of screening obtained by the end of season have to be confirmed at higher dose of fungus after repeating for one more season. Thus the method as described by Nene et al (1981), is highly time as well as space consuming and laborious.

It was observed in tobacco that the culture filtrate of F. oxysporium alone can mimic the action of the fungal infection in bringing about many of the wilt symptoms much earlier than the fungus itself, except vein clearing (Selvapandiya et al 1989). The toxicity of the CF was even found differential i.e. the plants susceptible to pathogen were also susceptible to the CF and vice versa (Selvapandiya et al 1989). We attempted to extend the usage of the said procedure in more important pulse crop like C. cajan because of simplicity, rapidity and ease of this screening procedure.

We have used liquid culture method to study the pathogenicity of fungi as well as to evaluate the relative susceptibility of varieties to the pathogen.

The symptoms like vein clearing and yellowing of lower leaves were visible 12-24 h after inoculation. The plants took 2-days for complete wilting when inoculated with CF or fungal mycelium. The undiluted CF was over active to give a good differential response between resistant and susceptible genotypes, whereas the diluted CF (15 % v/v) was differential in action. Appearance of the disease symptoms by the CF was noted much earlier than by the direct fungal infection. No blockage of vessels by the fungal mycelia were observed in the hand sections of the stem of plants grown in CF as compared with the plants infected with fungal mycelium. Thus these studies support the toxin theory of disease development originally proposed by Gaumann (1951).

Nene and Kannaiyan (1982) reported that the CF of F. udum was not toxic to pigeonpea seedlings even when the entire contents of suspension culture of the fungus was used as the inoculum. In the present studies it was found that 15% (v/v.) CF of F. udum was capable to mimic the action of fungal hyphae and spores in bringing about all the wilt symptoms much earlier than the fungus. Similar results were reported in tobacco by Selvapandiyan et al (1989). An effective

screening procedure has to be amenable to screen a large number of plants. It should be simple, rapid and significantly differential. The method used in the present investigation satisfies these criteria. Moreover, the screening could be carried out in a very small space. Similar reports of using CF to screen carnation plants for Fusarium (Buiatti et al 1985) and alfalfa cultivars for Verticilium (Iresand and Leath 1987) are available.

The technique standardized for screening pigeonpea var. T-15-15 against wilt disease using CF and toxin of F. udum was utilized for screening nine different varieties of pigeonpea. These varieties, viz. T-15-15, BDN2, AGS-498, Bandapalera, ICP-7336, GAUT 82-90, GAUT 82-99, NP (WR)15 and G-78-3 were subjected to 15 % (v/v) level of CF or 5% (w/v) fungus mycelium to determine differences in the susceptibility of varieties to the pathogen and its CF.All the varieties showed different levels of susceptibility of Fusarium wilt. The varieties were ranked based on their relative tolerance to the pathogen and its CF. Similar ranking of C. cajan varieties was observed in the conventional "sick plot" method used in agriculture to evaluate the wilt disease resistance of C. cajan to this

pathogen at the Model Farm, Gujarat Agricultural University, Baroda (personal communication). A number of other workers (Baldev and Amin 1974, Anonymous 1978-79, 1983-84, Nene et al 1980, Patel et al 1981, Nene and Kanniyan, 1982, Konda et al 1986, Zete et al 1986, 1987, Zote et al 1986) have also reported resistant or susceptible pigeonpea varieties to wilt in the field conditions. NP (WR)15, BDN2, Bandapalera and ICP-7336 were reported as resistant varieties and T-15-15 as susceptible variety against F. udum. Test tube screening using CF or toxin carried out in the present studies closely coincided with these results and pointed out that it coule be a very efficient method as compared to field screening of different pigeonpea varieties for wilt caused by F. udum.

In many cases the mechanism responsible for toxin or CF induced changes in plants is not known. The mode of action of toxin is considered as one of the most challenging and perhaps most fruitful aspects of toxin research (Misaghiu 1982). However, there are some reports regarding the consequences of pathogen infection on the host. These include permeability (Gaumann 1985, Wheeler and Black 1963, D"Alton and

Etherton 1984), irreversible damage to the chloroplast (Koruge 1978), changes in the rate of CO_2 uptakes (Duniway and Satyer 1971), alternations in photosynthetic rate (Steele et al 1976, 1978) and increase in the rate of respiration (Collins and Scheffer, 1958). A linear relationship was established between toxin concentration and various uncoupling activities in plant metabolism in tobacco by Selvapandiyan (1989).

Effect of CF on the destruction of chlorphyll was also studied by Selvapandiyan et al (1989). This prompted us to utilize chlorophyll degradation by CF as a method for screening pigeonpea varieties to wilt by leaf disc assay. Leaf discs (8 mm diameter) of susceptible var. T-15-15 were inoculated in various concentration of CF for dose determination in terms of chlorophyll degradation in culture. The optimum time period for exposure of leaf discs of CF was found to be 48 h. LD 50 was recorded at 5 % (v/v) CF. Utilizing this information, loss of chlorophyll by CF from the leaf discs of different varieties was examined. On the basis of loss of chlorophyll by CF, the varieties are rated susceptible to tolerant. A linear relationship was recorded between chlorophyll degradation by CF and susceptibility/tolerance

varieties to wilt. The destruction of chlorophyll in leaf-discs by CF was observed in alfalfa by Ireland and Leath (1989) and in tobacco by Selvepandiyan et al (1989). The results of the present investigation have further confirmed that leaf disc assay method could be effectively utilized for screening pigeonpea varieties against wilt.

It is obvious that somacional variation should rely on a powerful selection system to make it feasible for practical applications beside high regenerative system from the cells carrying desired genotype. In earlier studies of the present investigation, high frequency regeneration through somatic embryogenesis from cotyledonary explants was reported. There was a need to use the same system for screening different genotypic response to F. udum as well as for evolving genotypes resistant to wilt. The selection agent employed was CF of F. udum, incorporated into the culture medium used to obtain somatic embryogenic response from the cotyledonary explants.

In a preliminary experiment LD-50 dose of CF was determined. This dose was used as selection pressure for in vitro culture of cotyledons at all the stages till the development of complete

plantlets. The cotyledons of five varieties -two susceptible (T-15-15 and GAUT 82-90) and three resistant (Bandapalera, NP (WR)15 and BDN-2)-were subjected to selection pressure, i.e. LD-50 dose (20%, v/v) of culture filtrate. The highest embryogenic response was recorded in varieties resistant to wilt.

Hartmann (1984) demonstrated that alfalfa (Medicago sativa) regenerated from selected resistant cell lines expressed disease resistance. Some early encouraging findings are summarized in several reviews (Shepard 1981, Wenzel 1985, Evans and Sharp, 1986). The plants regenerated through somatic embryogenesis under selection pressure of LD-50 CF dose from cotyledons of five pigeonpea genotypes are yet to be tested under field conditions for their resistance to wilt. However, the selection pressure technique using cotyledonary explants demonstrated that it could be utilized for screening pigeonpea germplasm in addition to leaf discs assay method discussed earlier in this chapter.

Moreover, if any tolerant/resistant embryos are isolated it would offer an apportunity to raise plants there from and to process them through several generations to determine if the said resistant trait is inhereted.