Studies on Biochemical "Markers" to Screen Tobacco Mosaic Virus Disease Resistance/Tolerance in *Nicotiana tabacum* L.

Thesis submitted to

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for the award of Degree of

DOCTOR OF PHILOSOPHY

in

APPLIED BOTANY

By

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DECLARATION

I, **Subrahmanya K.N.,** hereby declare that the thesis entitled **"Studies on Biochemical "Markers" to Screen Tobacco Mosaic Virus Disease Resistance/ Tolerance in *Nicotiana tabacum* L."** embodies the results of bonafide research work carried out by me under the guidance of **Dr. M. Krishnappa,** Professor, Department of Applied Botany, Kuvempu University, Jnana Sahyadri, Shankaraghatta. I further declare that this or part thereof has not been the basis for the award of any other degree or diploma either in this or any institution or university.

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1. INTRODUCTION

Increasing the agricultural productivity through advanced technologies, without affecting the environment is a challenge of the century. Practice of sustainable agriculture i.e., farming that meets rising demands over the indefinite future at economic, environmental and other social costs, along with rising income, is the priority for the next few decades to overcome poverty and ecological imbalance.

At the present productivity level, and gradual decreasing tendency in land available for agriculture, it will be a stupendous task to meet the ever-increasing demand for agricultural produce in the years to come. Whenever, agriculture is practiced, pests and diseases also take a large share out of the yield. Diseases alone are reported to reduce the total harvest by about 12% (Day, 1993). A broad spectrum of crop diseases occurring throughout the world can, under appropriate conditions limit crop production. Since 1950's crops suffering from such diseases and pests have been controlled by the application of potent pesticides. However, long term and immediate health and environmental considerations due to the use of chemicals have enforced alternative methods of disease control. Concern for environment standards in advanced / developed countries, caused reduction in the use of pesticides. It has been now realized that chemicals are not the only weapons available to the battle. Combined use of hardy plants, crop rotation, tillage practices, biological controls and a minimal amount of pesticides collectively called integrated pest / disease management is the better approach.

1.1. Role of plant defense mechanisms in sustainable agriculture

The success of sustainable agriculture depends on enhancing the efficiency of bioconversions and the capacity to withstand adverse conditions in plants. In order to survive, green plants have developed a broad range of defense mechanisms viz., avoidance, tolerance and resistance to avert most of the pathogens / pests. Breeding for resistance using good and appropriate genetic resources is an achievable task. Though conventional plant breeding takes long time, it is estimated that about 1% yield increase annually is contributed through plant breeding (Plucknett and Winkelmann, 1995). Several varieties resistant to various pests and diseases have been successfully developed through plant breeding programmes.

1.2. Biochemical markers as tools in breeding programmes

During conventional breeding, selection of useful variants becomes difficult because, when plants are grown under natural conditions the expression of resistance is often suppressed. However, biochemical markers, which may or may not be part of the resistance mechanism, will be useful to breeders as a practical and reliable tool for predicting resistance to diseases, as well as to identify unexpressed desirable traits in crops. Adoption of standardized methods for each host pathogen interaction will underscore the influence of variability caused by biotic and abiotic factors on biochemical assays. This will help in reliable identification of the potential marker. The benefits of the use of biochemical markers are documented in several host-pathogen interaction systems. These biochemical parameters in addition to their use as markers may also help in understanding the role of genetic and metabolic factors in resistance mechanism. Biochemical markers for resistance possibly will eliminate time consuming,

extensive field trials and may expedite the breeding programme. These metabolic as well as genetic markers may provide an advanced approach to plant breeding and lead the breeding programmes in most advantageous direction.

1.3. Host pathogen system in the present study

Host pathogen interaction system of tobacco (*Nicotiana tabacum* L.) and tobacco mosaic virus (TMV), has been selected in the present study for possible identification of biochemical markers useful in breeding programmes.

Nicotiana is a genus where the evolutionary and phylogenetic relationships among species have been well worked out for more than 50 years by classical methods of taxonomy, morphology, cytology and genetics (East, 1928; Goodspeed, 1954; Kostoff, 1943; Smith, 1968). Since many years, tobacco has been used as a model system in bioengineering studies such as transformation that allows the introduction and expression of foreign genes. Though tobacco is one of the most studied plants much more is yet to be understood. Nuclear genome in *Nicotiana* is several times bigger as compared to that of *Arabidopsis* where sequencing of genes has been completed. Estimates suggest that about 27,000 different structural genes are expressed in tobacco leaves at any one time, and when one considers those genes unique to certain tissues or developmental stages; the total number of genes is probably closer to 60,000 (Goldberg *et al*., 1978; Kamalay and Goldberg, 1980). The complete chloroplast genome of tobacco was sequenced in 1986 (Shinozaki *et al*., 1986). Still a great deal of tobacco nuclear genome remains unexplored. The studies using randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and simple sequence repeats (SSR) indicated that tobacco is not highly polymorphic (Brandle and Bai, 1999).

1.4. Biology of tobacco

Though tobacco is a rich source of several phytochemicals like, nicotine sulphate, solanasol, organic acids, edible proteins and seed oil (Anon., 1989), at present the crop is used mainly for its narcotic values rather than for the alternative uses. The genus *Nicotiana* has 66 species. But *Nicotiana tabacum* and *N. rustica* are the only two species commercially grown. Tobacco is cultivated in at least 124 countries, occupying an area exceeding 4.3 million hectares (Anon., 2012). Tobacco is considered as one of the major employer and is the backbone of the economy of several countries including India, though its cultivation is restricted to 0.3 per cent of the world's arable land (Nagarajan, 1999). Thus, tobacco is the socio-economic lifeline of many developing nations. Continued international demand, versatility of the crop to grow in climates and soils non-suitable for other crops, hardiness of the crop to grow under limited moisture compared to other crops and higher employment opportunities are the advantages of tobacco cultivation.

1.5. Tobacco and national economy

In India, tobacco is a principal cash crop of great importance. It has been playing a prominent role in the development of national economy. Properties and usability of tobacco vary markedly with variety, locality, system of production and curing methods. Majority of tobacco types viz., Flue Cured Virginia (FCV), bidi, burley, *natu*, *cheroot*, hookah, snuff, chewing, cigar and *lanka* are cultivated in India, a unique feature, because of the prevailing varied agroclimatic zones. Commercial cultivation of tobacco is mainly concentrated in states like Andhra Pradesh, Karnataka, Gujarat, Maharastra, Bihar, Tamil Nadu and West Bengal, though cultivation is spread all over the country. Area under

tobacco cultivation is restricted to less than 0.3% of the arable land in India. But the country ranks 2nd in tobacco production and 3rd in exports. Tobacco contributes over Rs. 14,000 crores through excise revenue and more than Rs. 4,400 crores foreign exchange annually to the national exchequer. The crop provides employment to more than 34 million people in the country (Sarala *et al*., 2013). Though, India contributes around 10% of the total tobacco production in the world, Per capita consumption is only 0.8 kg and ranks 4th in total consumption, of which 19% is in the form of cigarettes (Anon., 2000).

1.6. International trade

Flue Cured Virginia (FCV) tobacco though has a share of less than 30% of total tobacco production and export from India, its share in terms of value is 60% in export and around 80% in excise earnings of the country (Anon, 2000). Farm prices of FCV tobacco in India is only 1.88 US $ per kg, which is far below the prevailing prices in USA (US $ 4.26), Zimbabwe (US $ 3.66) and Brazil (US $ 3.37) (Anon., 2014a). Thus, in international market, Indian tobacco is considered as "Value for money tobacco" (Nagarajan, 1998).

In FCV tobacco production, Karnataka is next to Andhra Pradesh and plays a key role in exports. More than 70% of the 70 to 85 million kg FCV tobacco produced in Karnataka is exported annually to West European, some African, Middle East and South East Asian Countries (Anon., 2014b). The naturally grown (rain fed) light soil FCV tobacco of Karnataka is considered as a superior neutral filler, having low chlorides, tobacco specific nitrosamines as well as pesticide residues and hence preferred in

international market. FCV tobacco (Fig. 1.1) is an important commercial crop without any sustainable alternatives cultivated under rain fed conditions in the light soil regions of the southern transition zone of Karnataka (Fig. 1.2). The sandy loam and loamy sand soils, lower diurnal temperatures, higher relative humidity and well distributed rainfall during the crop season (*Kharif*) are found congenial for the production of desired quality of FCV tobacco in this region.

1.7. Diseases of economic importance in tobacco

Like any other crop FCV tobacco encounters considerable threat from several diseases, pests and parasites. Unlike in major crops, here leaf is the product and has very rigid quality requirements. Hence control of diseases and pests that bring down the quality of cured leaf in addition to yield, is of great concern. Field crop diseases of economic importance in Karnataka include, black shank, brown spot, powdery mildew, frogeye and fusarium wilt caused by fungi, root knot caused by nematodes and tobacco mosaic caused by virus (Shenoi and Nagarajan, 1999).

1.8. Tobacco mosaic virus disease

Several viruses cause significant loss in tobacco every year world over. A survey of tobacco pests and diseases by a CORESTA task force (Delon *et al*., 1993) revealed that tobacco mosaic virus (TMV) was always an important problem in many tobacco producing countries. TMV is a very contagious disease that can occur in seed bed as well as in the field. It is one of the most infectious and persistent pathogen and is mechanically transmitted.

Fig. 1.1. Field view of Flue Cured Virginia tobacco crop in Karnataka.

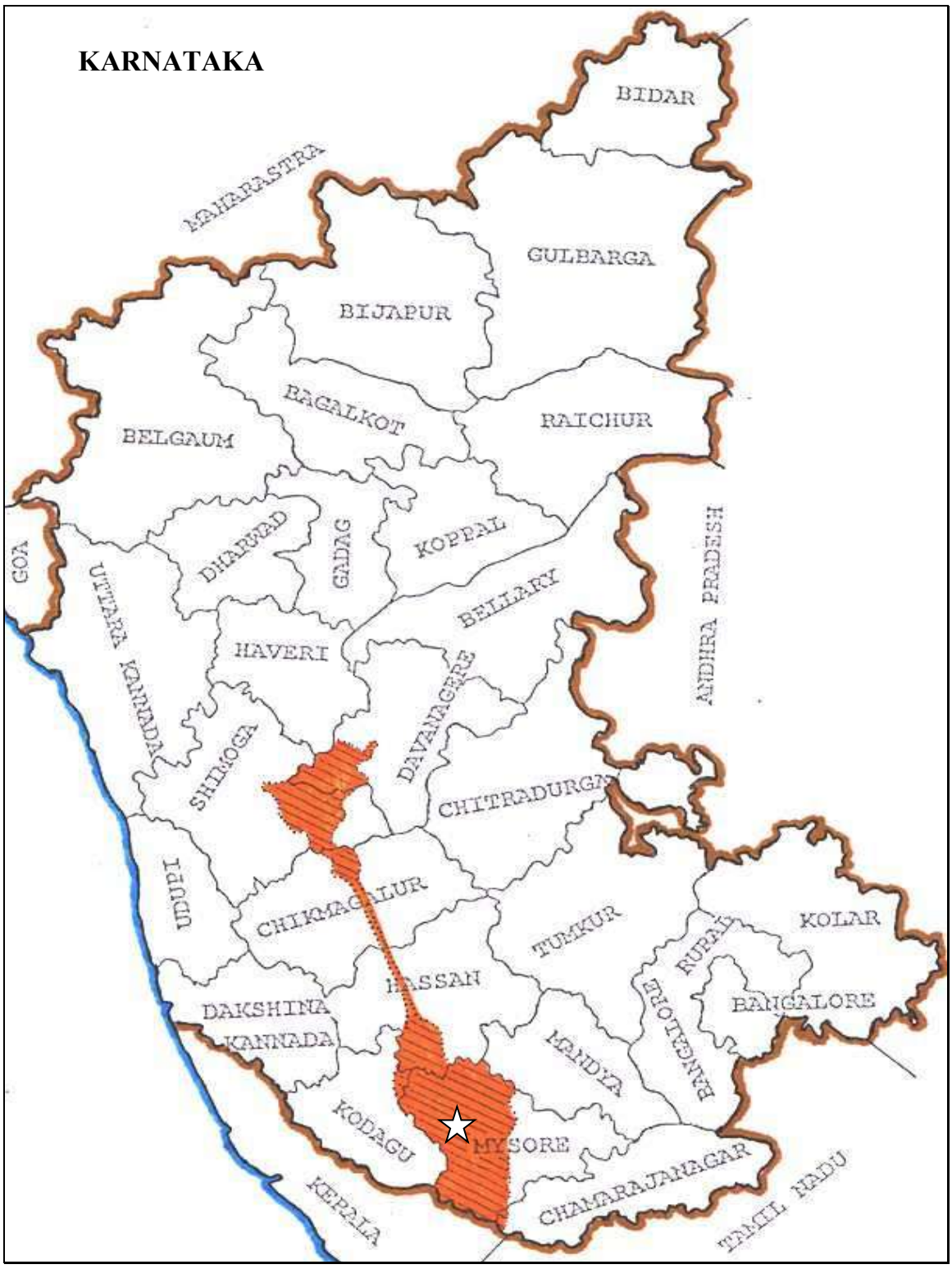


Fig. 1.2. FCV tobacco growing region in Karnataka ☆

Primary source of TMV inoculum can either be from tobacco products such as cigarettes, bidies, chewing tobacco, snuff etc. or from plant debris where the virus can survive for many years. Secondary spread is by workers during cultivation, hoeing, suckering and other field operations. There may be catastrophic outbreak, leading to complete loss of marketable crop, or they may be mild and relatively insignificant. Damage worth 2.8 million dollars to FCV tobacco due to TMV was reported in North Carolina, during 1966 (Russell, 1978). In North Carolina, 0.03% to 0.88% annual loss in value was estimated due to TMV (Johnson *et al*., 1983). Yield loss due to virus infection has been estimated to be about 1% in tobacco (Lewandowski and Dawson, 1994). TMV infections can change morphological, yield and quality characters. Plants infected early in the season are prone to higher yield losses (31.8%) as compared to late infection (21.8%) as indicated by the study in FCV tobacco in India (Reddy and Nagarajan, 1981).

1.9. Significance of tobacco mosaic virus

Tobacco mosaic virus (TMV) was the first virus to be crystallized in 1935 by Stanly and this became the landmark discovery creating new concepts about life. TMV protein was one of the first proteins of which the primary structure was understood. TMV played a key role in several studies on genetic code, nucleic acid replication and other cellular processes. TMV belongs to the tobamovirus group and virus particles or virions are straight tubes of 18 nm, having central hollow core of 4 nm diameter. About 2130 protein subunits and 6340 nucleotides are arranged in a right-hand helix around a single RNA molecule. The protein subunits have molecular weight of about 17,400. The RNA contains phosphate, D-ribose, two purine bases (adenine & guanine) and two pyramidine bases (cytosine and uracil) (Lucas, 1975).

Tobacco mosaic virus was shown to infect 199 of 310 species tested from 29 families (Lewandowski and Dawson, 1994). TMV induces classic symptoms on tobacco, with characteristic mottled appearance of the leaf with alternating areas of light and dark green patches, especially in younger leaves (Fig. 1.3). The sign of infection starts with vein clearing followed by vein banding and mosaic (Fig. 1.4), from which disease derives its name. In case of severe infections, plants are usually stunted (Fig. 1.5) and lamina of the leaf is considerably reduced in size giving a filiform appearance (Fig. 1.6). During the periods of high temperature and high light intensity, affected portions of the leaves may die and cause mosaic burn. Due to mechanical transmission, a series of plants in a row are likely to get infected by TMV (Fig. 1.7), causing significant loss.

1.10. Disease control

Tobacco mosaic virus related diseases have long been of major concern for tobacco growers, due to lack of efficient methods of disease control. Though, spraying virus inhibitors such as milk (Lucas, 1975), leaf extracts of *Basella alba, Bougainvillia spectabilis* and neem (Murthy and Nagarajan, 1986; Nagarajan *et al.,* 1993) have been reported to reduce mechanical spread of TMV, the most efficient way to control mosaic is to follow phytosanitory measures keeping the crop free from TMV. The use of resistant tobacco varieties could be recommended as the best tactic for managing TMV disease.

1.11. Disease resistance

The mechanism of TMV resistance found in *Nicotiana glutinosa* L. (Fig. 1.8) is through development of necrotic spots called local lesions (Holmes, 1938 and 1952). Necrotic lesions (Fig. 1.9) result from rapid death of a group of infected cells, with consequent localization of TMV in a small area. The necrotic response is attributed to hypersensitivity, where the rate of cell death is a rapid process, thus limiting the virus

Fig. 1.3. ***Nicotiana tabacum*** **leaf showing TMV induced mottled appearance and characteristic symptoms of light and dark green patches in comparison with healthy leaf.**

Fig. 1.4. Tobacco leaf showing first sign of TMV infection with veinbanding and mosaic.

Fig. 1.5. Tobacco plant with stunted growth due to sever infection of TMV.

Fig. 1.6: Filiform appearance of tobacco leaf due to malformation and reduction in size on TMV infection.

Fig. 1.7. Row of TMV infected plants due to mechanical transmission.

Fig. 1.8. *Nicotiana glutinosa* – a TMV resistant wild species of tobacco

Fig. 1.9. Necrotic lesion formation due to hypersensitive response for TMV infection in resistant variety.

Fig. 1.11. Variety Va 770-FCV tobacco cultivar having resistance to TMV infection.

spread. Resistance in *N. glutinosa* (also bred into *N. tabacum* cultivars Xanthi nc and Samsun NN) is reported to be N gene controlled (Holmes, 1938). Whereas, *N. Sylvestris* contains N' gene that localizes some tobamo viruses and TMV mutants, but not other viruses (Weber, 1951). Screening and evaluation of *N.tabacum* germplasm and several *Nicotiana* species for resistance have been performed in India and elsewhere (Lucas, 1975; Reddy and Nagarajan, 1981; Shenoi *et al.,* 1992). Nolla and Roque (1933) reported Ambalema type of resistance to TMV, which is controlled by 2 recessive genes (Clayton *et al.,* 1938). Valleau and Diachum (1941) concluded that resistance of the Ambalema type appears to be due to slow multiplication of TMV at the point of inoculation and slow release or complete failure of TMV release into the vascular tissue for long distance spread. But efforts to use this resistance in commercial cultivars failed because of the associations either via linkage or pleiotropic, with adverse effects on yield, agronomic traits and quality (Lewis *et al*., 2007). To understand the differences among the types of resistance, Fraser (1994) proposed the following schematic view (Fig. 1.10) of a replication cycle and the various possible targets of non-host, cultivar and acquired resistance mechanisms.

1.12. Constraints in breeding and selection of desired segregants

Studies of earlier researchers resulted in the identification of variety Va 770 (Fig. 1.11) which is resistant to TMV infection. However, this resistant variety produced poor quality leaves with off colour and lower grade index. The variety Va 770 was used as one of the parents in disease resistant breeding programmes. But the adverse traits of this variety were inherited even in the progenies, due to linkage of these traits with resistance as described by Blancard *et al.* (1999). Hence, the new varieties bred using Va 770 as parent lacked qualities required by the trade.

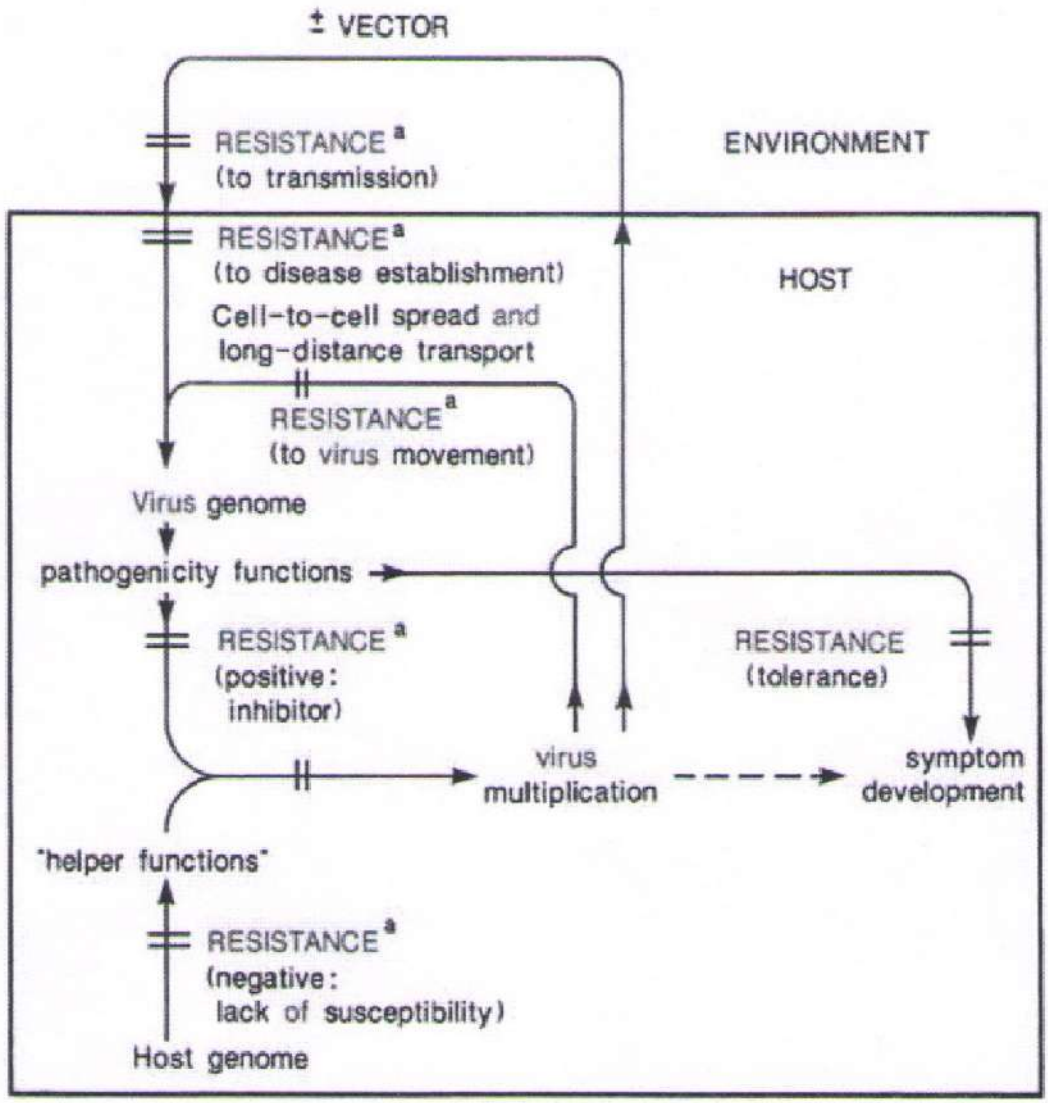


Fig. 1.10. The replicative cycle of plant virus and possible targets of cultivar resistance (Fraser, 1994).

In order to surmount the above constraint, breeding programme was initiated at CTRI Research Station, Hunsur. As a result, FCH 6248 variety was evolved, which showed tolerance to TMV at the same time produced not only better quality but also higher bright grade leaves (bright grade outturn), (Shenoi *et al.,* 1992). This cultivar FCH 6248 when challenged with TMV allowed entry of virus initially leading to symptoms of infection viz., mild mottling with chlorosis but the symptoms cleared

rapidly (Fig. 1.12) indicating prevention of spread of tobacco mosaic virus. Hence, this variety FCH 6248 had normal growth, yield and quality even though initially succumbed to TMV infection. This variety FCH 6248 developed at this research station still suffered disadvantage that the leaves did not meet some trade requirements like colour intensity, spotting and other manufacturing qualities, which called for further breeding work.

The objective of the future breeding work was to use FCH 6248 having tolerance to TMV as one parent and add required traits by crossing with a variety having desired yield and cured leaf qualities. By and large most of the varieties having commercially required traits are susceptible to TMV infection. In such breeding attempts one must ensure retention of the tolerant trait coupled with high grade out turn in the progeny. If acquiring of the desired traits can be detected by a rapid test at the early stage of the progeny, not only such a method will help in screening a large number of samples at a relatively short period of time but also helps in short listing the recombinants for field trials. In the present methodologies, the difference in the disease symptoms between the tolerant and susceptible plants among the segregants can be visualized late in the plant growth phase. Hence the retention / acquirement of the genes in the recombinant progeny can be known only at the later stages of growth to facilitate selections, requiring a minimum of 120 days (i.e., 60 days in nursery and 60 days in field after transplantation). If there could be a biochemical parameter to ascertain acquiring the desirable qualities and quantify tolerance, that marker can be used as a tool for early identification (30 days) and selection of desired recombinants among the segregants in the progeny. Such an early detection will therefore save breeding time because, as one need not wait until the late

Fig. 1.12. TMV tolerant variety FCH 6248 showing mild mottling and chlorotic symptoms on virus infection.

stage of plant growth to select the desired recombinant. This will also reduce the number of progenies to be tested in the laborious field evaluation. Such a study has therefore immense practical utility.

1.13. Present study

Developing markers for early detection of acquiring requisite traits amongst the recombinant progeny to improve the efficiency in breeding programme as described above was a long-felt need. Biochemical parameters such as induction / repression of certain key enzymes in plants can serve as tool to aid early detection of acquired qualities in the progeny. To achieve this, in the present study, levels of peroxidase, polyphenol oxidase, lipoxygenase, phenylalanine ammonia-lyase and ribonuclease enzymes as well as salicylic acid were estimated. These parameters were measured in three divergent plant systems namely (a) cultivar Va 770, (Fig. 1.13) resistant to TMV infection; (b) cultivar FCH 6248, (Fig. 1.14) tolerant to TMV infection; and (c) cultivar FCV Special, (Fig. 1.15) susceptible to TMV infection, to identify suitable biochemical marker if any. The reliability and usefulness of the selected biochemical marker was further tested by inoculating the virus to these plants and comparing the activities to those in controls without inoculation, or positive controls with mock inoculation wherein only mechanical injury was made without introducing the virus.

Fig. 1.13. A well grown plant of TMV resistant variety Va 770 selected for the study.

Fig. 1.14. Grown up plant of TMV tolerant variety FCH 6248 selected for the study.

Fig. 1.15. Adult plant of TMV disease susceptible variety FCV Special selected for the study.

1.14. Objectives

1. To measure the levels of enzymes peroxidase, polyphenol oxidase, lipoxygenase, phenylalanine ammonia-lyase as well as ribonuclease and salicylic acid in TMV disease resistant, tolerant and susceptible varieties of tobacco.

2. To compare the levels of these enzymes and salicylic acid in infection process in three divergent tobacco plant systems namely (a) cultivar Va 770, resistant to TMV infection; (b) cultivar FCH 6248, tolerant to TMV infection; and (c) cultivar FCV Special, susceptible to TMV infection.

3. To identify the resistance / tolerance specific marker useful in ascertaining the gene transfer during breeding programme.

4. To evaluate the usefulness of the selected marker/s in breeding programme.

1.15. Plan of work

1. Standardization of procedures and raising tobacco seedlings in laboratory.
2. Standardization of procedures and raising tobacco seedlings in shade house.
3. Optimization of procedures for extraction and assay of peroxidase enzyme in different varieties of tobacco before and after TMV inoculation.
4. Optimization of procedures for extraction and assay of polyphenol oxidase enzyme in different varieties of tobacco before and after TMV inoculation.
5. Optimization of procedures for extraction and assay of lipoxygenase enzyme in different varieties of tobacco before and after TMV inoculation.

6. Optimization of procedures for extraction and assay of phenylalanine ammonia-lyase enzyme in different varieties of tobacco before and after TMV inoculation.
7. Optimization of procedures for extraction and assay of ribonuclease enzyme in different varieties of tobacco before and after TMV inoculation.
8. Optimization of procedures for extraction and estimation of salicylic acid in different varieties of tobacco before and after TMV inoculation.
9. Standardization of procedures for isolation, maintenance/multiplication and inoculation of tobacco mosaic virus.
10. Effecting crosses to obtain recombinants for the inheritance study.
11. Standardization of procedures for protein estimation for enzyme quantification.
12. Defatting of seeds for lipoxygenase assay.
13. Standardization of procedures for polyacrylamide gel electrophoresis of seed protein.
14. Identification of biochemical tools to aid rapid selection of resistant / tolerant segregants and reduce the time taken in the traditional phenotypic symptom based selection process in breeding programme.

2. Review of Literature

2. REVIEW OF LITERATURE

2.1. Peroxidase

Peroxidase is widely distributed in the plant kingdom and several physiological functions are ascribed to it in plants. Peroxidase (POX) known as Donor: hydrogen peroxide oxidoreductase (EC 1.11.1.7) generally catalyzes a redox reaction between H_2O_2 as electron acceptor and many kinds of substrates. This enzyme differs from catalase mainly in the relative affinity to H_2O_2.

The increase in peroxidase activity has been studied in connection with oxidation of phenolic substances in diseased plants and resistance in the host was attributed to the toxicity of these oxidation products. Every infection process disturbs the enzymatic machinery of the infected tissues and the nature of this response may be specific to the disease concerned. Farkas and Kiraly (1958) reviewed the early works on fungal as well as several viral diseases in cabbage, citrus, potato, sweet potato, cotton and tomato, where, they have highlighted the role of oxidation process in host parasite relations.

The direct participation of peroxidase in the defense reactions of plants was reported by the Macko *et al.* (1968). These researchers observed peroxidase inhibiting mycelial growth in *Puccinia graminis* f. sp. *tritici*. The results of Daly and co-workers (1970, 1971) suggested that high peroxidase activity is a result rather than cause of incompatibility in wheat stem rust disease. Rudolph and Stahmann (1964) found an increase in peroxidase in resistant *Phaseolus vulgaris* plants but not in susceptible on infection with *Pseudomonas phaseoleae.*

Two groups of peroxidases on chromatographic and electrophoretic separation in potato were reported (Tomiyama and Stahmann, 1964). Even though, both the groups were found to increase by *Phytophthora* infection, significantly higher levels was noted in one group. Studies of Kedar (1959) showed a positive correlation between peroxidase activity and degree of resistance in potatoes to late blight. Elevated peroxidase activity lead to higher rate of necrosis and resistance was correlated to the resultant blockage of spread of the pathogen. Kedar (1959) also proposed peroxidase test as a tool in selection of potato varieties resistant to late blight. Barbara and Wood (1972) correlated peroxidase activity with cucumber mosaic virus multiplication and symptom development.

Studies on peroxidase in tobacco

Role of peroxidase in tobacco has been extensively studied. Lovrekovich *et al.* (1968) found a positive correlation between the level of peroxidase activity in tobacco leaves and resistance to *Pseudonomas tabaci*. The injection of heat-killed cells of pathogen into tobacco leaves enhanced peroxidase activity, induced formation of new isozymes and increased resistance to the same pathogen. Even the cell-free extracts of the pathogen could induce similar reaction. The participation of peroxidase in the defense mechanism was demonstrated by injecting solution of commercial peroxidase into the leaves, which resulted in increased resistance to disease.

Veech (1969) could not find any localization differences between black shank susceptible and resistant tobacco plants. The resistant plants were moderately diseased and the isozymes of peroxidase in this plant responded in a fashion similar to the susceptible variety. The failure to detect difference in peroxidase activity is attributed to the selection of plants having infection of stelar tissues.

The peroxidase activity was correlated along with other enzymes in relation to blue mould resistance (Wyatt *et al*., 1991). The activities of peroxidase and disease resistance were higher in old leaves than young ones. Lagrimini (1991) found a correlation between peroxidase activity and wound induced browning thereby suggesting a role in protecting the plants.

Isozymes of peroxidase

Viruses offer a unique opportunity to evaluate host enzymes precisely after infection because the enzymes are not present in the viruses themselves.

Virus infection leading to local lesion development resulted in the formation of new peroxidase components. A comparison of the change in the peroxidase isozyme spectrum in various host virus combinations indicated that mainly the host tissue determines the type of change and not the virus. It was proposed that cell necrobiosis accompanied by lesion development triggers in some way the formation of new proteins, including new peroxidase isozymes (Solymosy *et al.,* 1967).

Polyacrylamide disc electrophoresis of leaf extracts from different host parasite interactions viz. *Vigna sinesis* with cucumber mosaic virus (CMV) or tobacco ring spot virus (TRSV); *Phaseolus aureus* with southern bean mosaic virus, TRSV or TMV and *Nicotiana tabacum* cv Ky. 12 with tobacco streak virus or TMV, indicated quantitative, but not qualitative changes in peroxidase induction. An increase in activity of some of the isoperoxidases induced by infection differed from the ones increased due to resistant necrotic reaction. In tobacco at higher temperature, TMV induced, systemic chlorosis,

and at lower temperature local necrosis. The changes observed in peroxidase level were more pronounced when infection resulted in necrosis (Novacky and Hampton, 1968).

In tobacco - tobacco mosaic virus interaction, Simons and Ross (1970) noticed gradual increase in peroxidase activity, which attained 2.7 times that of the activity in the non-inoculated controls at 3 days after inoculation. Increased peroxidase activity coincided with lesion appearance. Significantly increased activity of the enzymes in upper leaves was recorded 4 to 5 days after inoculation on lower leaves. These leaves were not susceptible to TMV infection thereby suggesting that initial infection of TMV induced local resistance (Simons and Ross, 1970) in hypersensitive tobacco genotype Samsun NN. This increased activity of peroxidase is associated with resistance.

Weststeijn (1976) reported the existence of a gradient of peroxidase activity from the lesion edge to the interior tissue between the lesions in tobacco inoculated with TMV. Enhanced enzyme activity was mainly in the cells close to the necrotic lesion. It was observed that higher the lesion size higher was the enzyme activity. Based on the results a hypothesis has been proposed that, peroxidase activity has a central role, not in determining rapidity of necrosis, but in forming an “inducing” agent, which after movement to neighboring cells elicits the resistance.

Systemic acquired resistance of *Nicotiana tabacum* cv Samsun NN to tobacco mosaic virus manifested as decrease in lesion size is reported to be associated with increase in initial peroxidase activity. van Loon (1976) reported higher peroxidase activity with the increased lesion density on inoculated leaves. However, initial inoculum concentration and the leaf position on the stem did not influence the resistance.

Isozyme fingerprint studies (Lagrimini and Rothstein, 1987) indicated tissue specificity in *Nicotiana tabacum.* Infection of tobacco with tobacco mosaic virus induced two moderately anionic isozymes in the leaves in which virus was applied. Similar induction was also noticed in upper uninoculated leaves of the same plant. This type of systemic response was not found by wounding the leaves. Virus infection induces isozymes of peroxidase that are different from those induced by wound. These results indicated that peroxidases are certainly important in the reinforcement of cell wall. Elevated levels of two isozymes P 56 and P 61 have been considered as reliable markers for TMV infection.

High resistance to different diseases in the hybrid *Nicotiana glutinosa* x *Nicotiana debney* prompted Goy *et al.* (1992) to study the level of enzymes in it. They observed the resistance of the hybrid was associated with high levels of chitinases, β-1,3 glucanase, peroxidase and polyphenol oxidase. All the acidic isozymes of peroxidases produced after infection in both the parental species were constitutively expressed in the hybrid. Thus, peroxidases along with hydrolases are presumed to be involved in the resistance against various pathogens including viruses and are also known to be inherited by the hybrid to express resistance to the pathogen.

Sheen (1969 & 1970) studied on distribution of peroxidase in different plant parts of tobacco, *Nicotiana tabacum.* Peroxidases in leaves of 60 *Nicotiana* species, 19 cultivars, autopolypliods, inter specific hybrids, and amphidiploids have been compared by polyacrylamide gel block electrophoresis. At least 19 peroxidase bands of which, four cathodic and 15 anodic were detected from different species. The number varied from two bands in *N. arentsii* to 12 bands in *N. tabacum.* Specific differences and varietal

resemblance in root peroxidase bands were also observed in nine species and 20 varieties analyzed. Zymograms from autopolyploids and amphidiploids appeared to be identical to that of diploid parents, suggesting that peroxidase-banding patterns are independent of ploidy levels. The failure of dissociating peroxidases into subunits and forming hybrid enzymes in the interspecific hybrids indicate that peroxidases may be single molecular entities and are governed by multiple genes in the genus *Nicotiana.* The additive pattern of peroxidase zymograms in inter specific hybrids suggest dominant genes and/or codominant alleles at all loci involved for peroxidase synthesis. Some species classified in different sections or subgenera but having a common geographic center of origin showed close similarities in peroxidase zymogram suggesting thereby that the species closely related in phylogeny, and / or geographic isolation changes the peroxidase genes through mutation and selection.

Electrophoretic patterns of seedling root peroxidases were studied by Smith *et al.* (1970) to understand the genetic relationship among 61 species of the genus *Nicotiana*. In all, 33 peroxidase bands were identified and no single specific band was common to all *Nicotiana* species. Hypergeometric distribution model indicated that, the probability of observed band matching due to chance was less among species within a section. This indicates a closer genetic relationship in agreement with the established taxonomy of the genus. Comparison of peroxidase band pattern of 17 amphiploids indicated 75% similarity among the bands of amphiploid with one or both parents, while 25% were possibly new (hybrid) bands.

Sheen (1983) reviewed isozyme variation including peroxidase among and within *Nicotiana* species as well as isozymes in cell and tissue cultures. Biological application and future direction of isozymes were also discussed in this review.

Use of peroxidase as a tool to identify resistance in breeding

Studies by Reuveni *et al.* (1991) on levels of peroxidase in lettuce and associated field resistance to downy mildew suggested that imposition of 50% selection at F_2 on the basis of high peroxidase activity would result in retention of individuals yielding 80% of the most resistant F_3 progeny. This selection procedure could permit a 50% reduction in field trial size without drastically reducing the efficiency of selection. Based on these studies Reuveni (1995) suggested that the mechanism of disease resistance may be specific or non-specific, but the rapid recognition of the pathogen by the plant as virulent could be highly specific. In most host–pathogen systems, total peroxidase activity represents a marker for non-specific response. A rapid assay for monitoring peroxidase activity as a non-specific marker for primary selection was also suggested.

2.2. Polyphenol Oxidase (Catechol oxidase)

Polyphenol oxidase, known as catechol oxidase, diphenol oxidase or diphenol oxygen oxidoreductase (1.10.3.2) catalyzes the oxidation of *O*-diphenols to *O*-diquinones (Fig. 2.2.1). Catechol oxidase is widely distributed in plant kingdom, and the enzyme does not appear to be confined to any particular part of the plant. Its presence has been reported from a variety of plant organs and tissues. The level of catechol oxidase often changes markedly during the development of the plant and may be considerably altered by growth conditions.

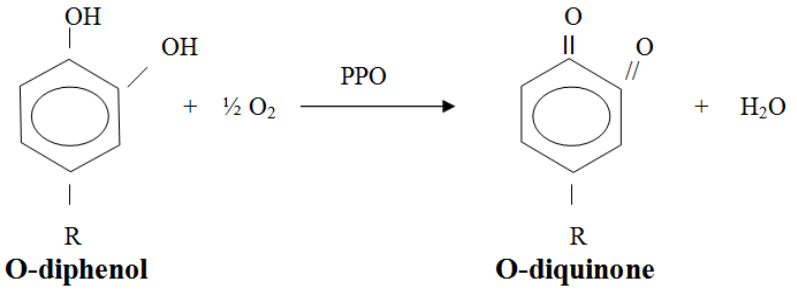


Fig. 2.2.1. Catalytic action of polyphenol oxidase enzyme

Detailed reviews by Mayer and Harel (1979), Vaughn and Duke (1984) and Mayer (1987) have brought out a comparative account of information available on polyphenol oxidases in plants. Catechol oxidases are identified to be located generally in chloroplasts, though there are reports of their presence in bundle sheath or mesophyll cells and thylakoids. The catechol oxidase is reported to be nuclear coded in *Nicotiana.*

Keeping in view of the following properties of the enzyme viz. the enzyme may be soluble or particulate and it often appears in several subcellular fractions, the phenomenon of latency; the activation of the enzyme under certain conditions due to conformational changes; the presence of native inhibitors; the enormous variation in enzyme levels at different periods of growth and development, the separation of the enzymes from most of its substrate due to cellular or sub cellular compartmentalization, any attempt to ascribe a physiological function to catechol oxidase in green plants is considered to be a formidable task (Mayer and Harel, 1979).

Polyphenol oxidase in wound healing and prevention of infection

Catechol oxidase is known to play an important role in browning of fresh fruits and vegetables. Following injury, plant tissues rapidly turn brown or black. The reaction

is catalyzed by catechol oxidation. The oxygen tension at the active site of the enzymes may increase, the sequestered phenols become accessible to the substrate, and latent enzymes may become activated. Possibly this enzyme promotes wound healing and also prevents or reduces infection.

There is a functional significance to the rapid production of quinones after injury (Butt, 1980; Mayer and Harel, 1979). Whether due to mechanical injury or cellular disruption from disease, the quinones produced by the enzymatic action are very reactive, making them good candidates for protection of plants from pathogens. Polyphenol oxidase is relatively a stable enzyme and reactivation of latent enzyme can take place over long time periods (Montalbini *et al.,* 1981), resulting in continued production of quinones. These findings have prompted many workers to study the possible function of catechol oxidases in relation to the plant pathogen interaction.

Kosuge (1969) in his review drew the following general conclusions. Catechol oxidase activity increases following infection by virus, bacteria fungi or mechanical injury. The increase may be due to solubilization and activation of latent enzymes of the host, or even due to *de novo* synthesis.

Necrosis associated with race-specific resistance is normally characterized by the formation of brown to black pigments (melanin) throughout the cell walls and collapsed protoplasts. Walls of adjoining live cells also may become melanized. The intensity of melanin formation often is greatest in highly resistant plants, suggesting that melanins or their precursors contribute to resistance (Bell, 1981). Melanins in plants are formed principally from various ortho-dihydroxy phenolic compounds. The enzyme polyphenol

oxidase and peroxidase oxidize the colourless dihydroxy phenols to give the coloured ortho-quinones (Mayer and Harel, 1979). The tannins and the ortho-quinones have some toxicity to most microorganisms and viruses, but more important are that; they inactivate extra cellular enzymes produced by microorganisms, which are needed to invade the host tissue (Hunter *et al.*, 1978).

In potato when the cut surface was inoculated with the incompatible *Phytophthora infestans* to which the tissue was resistant, polyphenol oxidase activity increased. In contrast, no increase was found when the inoculation was done with the compatible race to which the tissue was susceptible (Tomiyama, and Stahmann, 1964). These studies revealed that inability to induce polyphenol oxidase could lead to host susceptibility.

Hampton and Fulton (1961) from their *in vitro* studies inferred that the action of polyphenol oxidase causes instability of necrotic ring spot viruses in prune dwarf and sour cherry. Sharma *et al.* (1990), attributed post infectional increase of polyphenol oxidase, high activity of peroxidase and acid phosphatase in tomato roots to resistance against *Meloidogyne incognita*. In contrast, Barbara and Wood (1972) in their work with cucumber plant observed a rise in polyphenoloxidase level in cultivar Ashley, but could not correlate with the decline in virus infectivity. Similarly, Pryor (1976) reported non-involvement of catechol oxidase in rust resistance of *Zea Mays*.

Role of polyphenol oxidase in tobacco

In flue-cured tobacco, colour of the cured leaf plays an important role in deciding the grade and market preferences. Polyphenoloxidases are of great importance in tobacco,

as oxidation of polyphenols catalysed by this enzyme during curing causes browning and reduces market quality of bright leaf tobacco (Johnson, 1966; Watkins and Hassler, 1962). Production of cherry red off coloured tobacco has been a problem, which is usually confused with orange-red colour. Weeks *et al*. (1993) by *in vitro* reaction have shown that, polyphenoloxidases from green tobacco leaves oxidized chlorogenicand caffeic acids to *O*-quinines, which reacted with nornicotine to produce red colour thereby lowering the leaf quality and marketability.

The studies of Sheen (1973) and Stafford and Galston (1970) indicated tissue related isozyme specificity of polyphenol oxidase isozymes in tobacco. Polyphenol contents of 46 cultivars including 11 tobacco mosaic virus disease resistant genotypes were estimated by Chakraborty and Gopalam, (1979). These authors reported resistance to tobacco mosaic virus is found to be independent of levels of polyphenols in tobacco.

In tobacco cultivar Samsun NN, van Kammen and Brouwer (1964) observed induction of polyphenol oxidase with in seven hours after TMV inoculation, although increase could be measured up to 2 to 3 days after inoculation. The increase in enzyme activity was found in the entire leaf without restricting to infected necrotic tissues alone, indicating the enzyme has no relationship between local lesion formation. There appeared no qualitative differences between the enzyme before and after infection with TMV. The enzyme after infection is hence presumed to be plant specific.

Simmons and Ross (1971) could detect the activation of polyphenol oxidase activity within a day after inoculation and was greater in challenged resistant than in challenged non-resistant leaves in cultivar Samsun. Although polyphenol oxidase activity appeared to increase significantly prior to lesion formation, they inferred the need for a

more critical study with greater number of sampling to establish the role. Increase in polyphenol oxidase activity was significant in upper leaves of Samsun NN tobacco and associated with the induction of systemic resistance following inoculation of lower leaves with tobacco mosaic virus.

From the results described above, it is not possible conclusively to assign a direct role for polyphenol oxidase in plant disease resistance, even though the enzyme plays an indirect role.

2.3. Lipoxygenase

Lipoxygenase (LOX) (linoleate: oxygen, oxido-reductase, EC 1.13.11.12) is a dioxygenase that catalyzes, as a primary reaction, the hydroperoxidation, by molecular oxygen of linoleic acid and other polyunsaturated lipids that contain a *cis, cis*-1, 4-pentadiene moiety to produce conjugated hydroperoxydiene derivatives (Fig. 2.3.1).

Presence of lipoxygenase activity (then termed 'lipoxidase') in plants was first reported about 70 years ago (Andre and Hou, 1932). The physiological role of the enzyme is still not fully understood, though a species of LOX from soybean was one of the first enzymes to be crystallized successfully (Theorell *et al.,* 1947). Lipoxygenases are particularly relevant to food plants because, their action destroys the essential polyunsaturated fatty acids and produce hydroperoxide that can decompose to form derivatives with characteristic tastes and odors (both desirable and undesirable). Lipoxygenase has been reported in several organisms including higher plants (Eskin *et al.,* 1977 and Vick and Zimmerman, 1987). Schewe *et al*. (1986), Mack *et al*. (1987), Vick and Zimmerman (1987), and Siedow (1991) have reviewed the works on lipoxygenase.

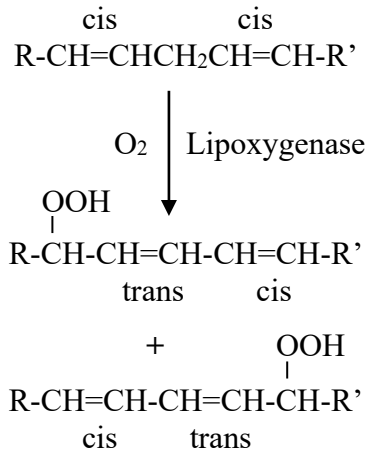


Fig. 2.3.1. Lipoxygenase catalysed hydroperoxidation of polyunsaturated lipids to produce conjugated hydroperoxydiene derivatives

The amount of lipoxygenase activity found in a given plant tissue was correlated with its rate of elongation (Siedow, 1991) implying a role in plant growth. The lipoxygenase-mediated pathway gives the end products like traumatin a "wound hormone" and Jasmonic acid, important in growth regulation (Vick and Zimmerman, 1987; Vick and Zimmerman, 1983). The role of plant lipoxygenases in wounding and other related stress has been suggested by Galliard (1978).

Increase in lipoxygenase activity soon after germination and during early stages of seedling growth has been reported for a number of species including rice (Ohta *et al*., 1986), wheat (Guss *et al*., 1968) mustard (Oelze-Karow *et al.,* 1970), lupine (Beneytout *et al.*, 1988) cucumber (Matsui *et al*., 1988) and pearl millet (Nagarathna *et al*., 1992). A common feature in all the studies is increase in lipoxygenase activity in the developing seedling with in the first 50-72 hours after germination, followed by decline in activity over subsequent 48 hour.

Works on plant pathogen interactions indicated a correlation between the levels of lipoxygenase activity and degree of resistance in many crops. Lipoxygenases are known to generate singlet oxygen during the reaction (Kanofsky and Axelrod, 1986). This oxidative burst is correlated with the hypersensitive reaction in a number of plant pathogen interactions and may be playing an important role in disease resistance. Treatment with methyl jasmonate was shown to induce the expression of proteinase inhibitors in tobacco and alfalfa (Farmer and Ryan, 1990). Thus, jasmonic acid, one of the end products of lipoxygenase-mediated reaction may also serve as a signal transducer activating defensive genes in plants.

Yamamoto and Tani, (1976) identified the induction of two new LOX isozymes and increase in the LOX activity during incompatible interaction in oat plant on crown rust pathogen infection leading to resistance. But, such increase was not observed during compatible interactions in susceptible variety, making it vulnerable to disease.

In rice plant, infection of *Pyricularia oryzae* induced rapid increase in LOX activity from first day of inoculation in resistant rice leaves, but the activity showed a declining trend after 3 days (Namai *et al.,* 1988). Higher the resistance, higher the enzyme activity has been reported.

Presence of LOX 1, -2, -3 isozymes in leaf tissues of rice was reported (Ohta *et al.* 1991). The activity of leaf LOX-3 in particular increased in the leaves infected with incompatible race of blast fungus. 13-L-hydroperoxy-9, 11 (Z, E)-Octadeca dienoic acid produced from linoleic acid and 13-L-hydroxy-9,11 (Z, E)-octadecadienoic acid were found to be highly inhibitory to the germination of conidia of fungus.

From the study using ethylene generating chemical ethephon in rice, Sekizawa *et al*. (1990) proposed that induction of LOX in leaves infected with pathogen is dependent on the generation of endogenous ethylene. Presence of activated O_2 is essential for the induction of ethylene.

In pearl millet, Nagarathna *et al.* (1992) reported higher enzyme activity in seed of downy mildew resistance genotypes and proposed LOX activity of seed can be a biochemical marker for ascertaining degree of resistance in screening different genotypes of pearl millet. On zoospore inoculation, the LOX activity increased in resistant genotypes and not in susceptible, showing the possibility of significant role of LOX in resistance mechanism.

Keppler and Novacky (1987) reported enhanced lipid peroxidation during the hypersensitive resistance reaction in cucumber cotyledons following infection with *Pseudomonas syringae* pv *pisi.* They suggested that during the hypersensitive reaction leading to necrotic lesion formation, the membrane lipids are altered due to lipid peroxidation catalyzed by LOX.

In their studies on soybean, Pfeiffer *et al*. (1992) showed isolines lacking LOX2 in seed exhibited higher susceptibility to the *Phomopsis longicolla* than the control with LOX2 activity. Absence of both LOX2 and LOX3 significantly increased the susceptibility.

Yabuuchi *et al*. (1982) have developed an enzyme linked immunosorbent assay (ELISA) for the quantitative determination of Lipoxygenase-1 & -3 in soybean, which

has considerable potential for screening of genotypes deficient in specific lipoxygenase isozymes in segregating soybean populations.

Two cDNA clones that represent lipoxygenase genes designated as LOX 7 and LOX8 in soybean, that display increased expression in leaves following wounding were isolated by Saravitz and Siedow (1996). High levels of expression of both genes were detected after wounding in young leaves, flowers and immature seedpods, in addition to the reaction to methyl jasmonate treatment. They have suggested that lipoxygenases encoded by these genes may participate in general physiological processes that are enhanced following physical damage.

Croft *et al*. (1993) reported in *Phaseolus vulgaris* evolution of several lipid derived volatiles including the ones from linoleic acid, as a hypersensitive resistance response to *Pseudomonas* sp. This type of activation of "lipoxygenase pathway" was not observed either in buffer inoculated leaves or in pathogen inoculated susceptible interaction, suggesting the specificity to incompatible interaction. The results clearly indicate that elicitation of enzyme and production of lipid derived volatile compounds are confined to resistant reaction.

Isolation and characterization of an *Arabidopsis* LOX-1 cDNA clone was attempted by Melan *et al*. (1993). They reported the induction of this gene in response to hormones, abscisic acid and methyl jasmonate, as well as attack by *Pseudomonas* pathogen suggesting a role for LOX in plant defense.

The treatment of arachidonic acid, an elicitor of hypersensitive reaction, induced hypersensitive cell death and browning in LOX-active calli of *Solanum tuberosum*, but

not in LOX-null calli. This result indicated that LOX activity helped in peroxidation of arachidonic acid, thereby inducing hypersensitive cell death (Vaughn and Lulai, 1992).

Farmer and Ryan (1992) showed the participation of octadecanoid precursors of jasmonic acid i.e., linoleic acid, 13(S) – hydroperoxy linolenic acid and phytodienoic acid in lipid-base signaling system that activates proteinase inhibitor synthesis in tomato leaves. They also proposed a model in which systemin and oliogouronides produced by insect or pathogen attack interact with receptors in target cells to initiate octadecanoid-based signaling pathway, which induces expression of proteinase genes.

Studies carried out by Somnath and Mahesh (2013) revealed that the incompatible interaction between pathogen *Fusarium oxysporum* f. sp. *ricini* and host Castor triggered LOX activity in leaves and root. They suggested the possible influence of hyper sensitive reaction for elicitation of enzyme.

Role of lipoxygenase in tobacco

Specific signal and defense responses associated with the interaction between tobacco and certain races of *Phytophthora parasitica nicotianae* were reported by Esquerre'-Tugaye' *et al*. (1989). They proposed a hypothesis that elicitor recognition induces membrane depolarization and ethylene synthesis, which in turn might play a role as cell-to-cell signals. Intracellular signals might involve phospholipid metabolites derived by lipoxygenase-mediated reaction. This gives a new dimension to the role of LOX in pathogenesis.

Rickauer *et al*. (1992) studied the regulation of proteinase inhibitor production in tobacco cells by elicitors prepared from cell walls of *Phytophthora parasitica* var.

nicotianae. The results indicated methyl jasmonate and not ethylene is a signal molecule for elicitation of proteinase inhibitors. Methyl jasmonate is derived from hydroperoxides. These hydroperoxides in turn are obtained, by the action of lipoxygenase on polyunsaturated fatty acids. Since lipoxygenase is also induced in elicitor-treated tobacco cells, the results indicated a role for this enzyme in the regulation of plant defense reaction.

Lipoxygenase was induced in suspension cell culture of tobacco cultivar Wisconsin and seedlings of Kentucky -15 by treatment with glycopeptide elicitors prepared from cell walls of *Phytophthora parasitica* var. *nicotianae*. The enzyme with molecular weight of about 96,000 Da was purified and identified as type 2 lipoxygenase (Fournier *et al*., 1993).

Studies on cDNA sequences for lipoxygenase isozymes indicated, the c-terminal 9 amino acids of the plant lipoxygenases sequenced to date are identical with an exception of tobacco. In tobacco, no sequence similarity was seen with 9 amino acids and an additional 20 amino acids are added on to the terminus (Bokjans *et al*., 1988). Thus, the antibody for isozymes of other plants may not be of use as a biochemical tool in tobacco.

Changes in the lipid profile of host cell membranes were characterized during the hypersensitive reaction induced by *Pseudomonas syringae* pv. *syringae* in plants of *Nicotiana tabacum* (Adam *et al*., 1989). Before the appearance of symptoms of hypersensitive reaction, increase in $O_2^{\cdot -}$ generation and lipid peroxidation was detected; implying an important role to the LOX in the pathogen induced hypersensitive reaction.

Doke and Ohashi (1988) showed a marked O_2^- generating activity on inoculation with TMV in leaf discs of tobacco cultivar Samsun NN, a TMV resistant genotype. Leaves of non-infected Samsun NN and those of TMV-infected and non-infected Samsun nn (susceptible) cultivar showed little O_2^- generating activity. The technique of using nitrobluetetrazolium served as a useful histochemical assay for active oxygen species. The results lead to propose that activation of an NADPH-dependent O_2^- generating reaction may be involved in the induction of the necrotic lesions on TMV infection in leaves of resistant cultivars. O_2^-generation in biological systems is known to generate other derivative superoxide molecules. These molecules in turn may stimulate lipid peroxidation leading to cell necrosis. Hence, LOX has a role in the manifestation of pathogenicity.

Kunstler *et.al*., (2007) studied the variance in activities of LOX and Glutathione S-transferases (GST)/Glutathione peroxidases (GPOX) in TMV-inoculated tobacco plants to understand the alterations in the fatty acid hydroperoxide metabolism. They demonstrated the marked early increase of LOX activity in cell free extracts in resistant genotype (Xanthi-nc) post infection, while the induction was weaker in susceptible (Samsun-nn) and attributed LOX to play an important role in plant disease resistance. The elevated GST/GPOX activity was suggested as protective mechanism around the infection site. They opined that the TMV-elicited oxidative stress is stronger in resistant than in susceptible leaves.

2.4. Phenylalanine Ammonia-Lyase

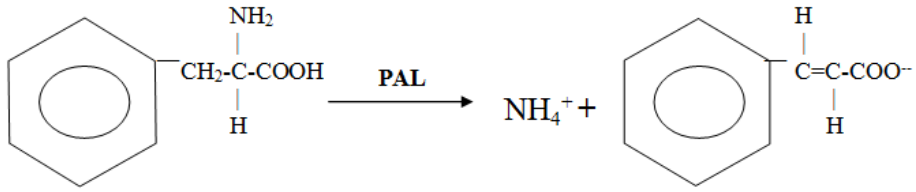
Phenylalanine Ammonia-Lyase (PAL) (E.C.4.3.1.5) enzyme is an integral part of secondary metabolism in plants. The enzyme attracted the attention by plant physiologists, biochemists and phytochemists, because of the significant fluctuation in its level at relatively short interval of time in response to a wide variety of stimuli. Cinnamic acid, one product of the enzyme reaction, is the precursor of a large variety of secondary constituents, some of which, e.g., lignins, may account for an appreciable proportion of the total carbon in a plant.

The hypersensitive response of plants to pathogens has often been reported to result in cell wall thickening, which impairs manifestation of the pathogen through plant tissues. Lignin and lignin like polymers derived from the phenyl propanoid pathway-involving PAL are thought to play a major role in the building up of these mechanical barriers.

Plants are also known to produce phytoalexin as a defense mechanism. Kuc (1972) considered the term phytoalexin in a broad perspective under which chemical compounds contributing to disease resistance can be classified whether they are formed in response to injury, physiological stimuli, the presence of infectious agents or the products of such agents. One of the key enzymes implicated in the formation of such phytoalexins is PAL.

Phenylalanine ammonia-lyase catalyses the deamination of *L*- phenylalanine to yield trans-cinnamic acid and ammonia (Kuokol and Conn, 1961) (Fig. 2.4.1). The enzyme has been found in all green plants including the higher cryptogams, the

basidiomycetes, and streptomyces (Camm and Towers, 1973). PAL isolated from manyorganisms has been characterized, including the one from tobacco (O'Neal and Keller, 1970).



Phenylalanine **Cinnamic acid**

$$NH_4^+ + OH^- \longrightarrow NH_3 \uparrow + H_2O$$

Fig. 2.4.1. Enzymatic action of phenylalanine ammonia-lyase on phenylalanine

O'Neal and Keller (1970) have demonstrated the presence of PAL in the leaves of *Nicotiana tabacum* var. Burley 21 and the enzyme has been purified approximately 200-fold. They found that the general properties of tobacco PAL are rather similar to the ones isolated from other higher plants. The optimal pH for the reaction is 8.0 to 8.6. The enzyme did not respond to substrates like *D*-phenylalanine, *L*-tyrosine, *M*-tyrosine, *O*-tyrosine and dihydroxy phenylalanine, thus proving substrate specificity to L-phenylalanine. The enzyme inhibition by cinnamic acid in a competitive manner suggested that trans-cinnamic acid might play a role in regulating *in vivo* biosynthesis of phenolic compounds in tobacco.

In tobacco as well as in many other plants, the incompatible host parasite interaction is characterized by the induction of various defense mechanisms to restrict infection. One common feature of many incompatible interactions is the development of

a hypersensitive response on the plant. Symptoms of the hypersensitive reaction are collapse and death of plant cells in and around infection sites. The hypersensitive reaction coincides with numerous metabolic changes of the affected plant cells. Increase in the enzyme activity is one such change (Bell, 1981). Enzymes of phenylpropanoid pathway, especially phenylalanine ammonia-lyase is found to be a good biochemical marker of necrotic reaction. PAL is also found to be a key enzyme in salicylic acid mediated systemic acquired resistance hypothesized in tobacco (Shulaev *et al*., 1995).

Green *et al*. (1975) reported increased activity of PAL in wheat lines in response to infection with *Erysiphe graminis* f.sp. *tritici*. Though increase in PAL activity was observed in both susceptible as well as resistant genotypes, the activity of PAL reached the basal level at 48 hours post inoculation in susceptible genotypes. In resistant genotypes the activity continued to increase beyond 48 hours and reached peak at 96 hours. Histochemical studies indicated the accumulation of lignin at 96 hours after inoculation only in resistant genotypes.

Moerschbacher *et al*. (1989) showed increased activities of PAL in wheat inoculated with *Puccinia graminis*. The activation of PAL coincided with hypersensitive reaction. Hence, the authors suggested that the differences between compatible and incompatible interaction might be the timing of induction of enzymes involved in lignification.

In barley, PAL activity and cinnamic acid synthesis increased by four hours post inoculation with *E. graminis* (Shiraishi *et al*., 1989) suggesting their involvement in host pathogen interaction.

Nagarathana *et al*. (1993) observed elevated activity of PAL, 24 hours post inoculation in resistant but not in susceptible genotypes of pearl millet on inoculation with pathotype 1 of *Scelrospora graminicola*. The increase in the activity was restricted only to shoot portion. Histochemical tests for lignin were positive in infected cells in the resistant genotypes.

A biphasic induction of PAL i.e. initially at 12 hours followed by the second at 35 hours from inoculation as a resistance reaction to *E. graminis* infection was observed in Oat (Yamamoto *et al*., 1977). The first phase of induction was common to both susceptible and resistant genotypes.

The second phase of PAL induction is of significance in resistant reaction. Studies on wheat and stem rust interaction (Moerschbacher *et al*., 1988) also revealed two peaks in PAL activity. The first increase was common to both resistant and susceptible lines. The second phase of increase was noticed only in resistant genotype at the time of hypersensitive resistance reaction.

In maize, Dickerson *et al*. (1984) could not record any increase in PAL levels either in susceptible or resistant genotypes when inoculated with *Helminthosporium maydis*. While Pascholati *et al*. (1985) have reported induction of PAL activity in compatible interaction and not in incompatible interaction of maize plant with *Helminthosporium maydis* as well as *H. carbonum*.

Through immuno histochemical techniques *de novo* synthesis of PAL in immature pea pods inoculated with macroconidia of *Fusarium solani* was reported by Loschke

et al. (1981). In cowpea, a virulent race of *Phytophthora vignae* induced PAL activity both in resistant genotype and also in susceptible cultivars (Ralton *et al*., 1988).

Several workers have reported enhanced PAL activity as a result of incompatible interaction between soybean with *Phytophthora megasperma* f. sp. *glycinea* (Borner and Grisebach, 1982; Battacharyya and Ward, 1988). Esnault *et al*. (1987) have reported the increase of m RNAs for PAL in addition to the increase in PAL in an incompatible interaction, but not in compatible interaction.

In resistant cultivar *Ulmus pumila* PAL activity increased 16 folds in response to spore treatment of pathogen *Ceratocystes ulmi* to cell culture. But in susceptible species *U. compestris,* PAL activity remained unaltered (Corchete *et al*., 1993). Incompatible virus host interactions inducing PAL activity has been reported in many solanaceous crops including tobacco.

Enhanced activity of PAL in resistant potato tuber discs inoculated with *Phytophthora infestans* has been reported by Friend *et al*. (1973). Increase in PAL activity in resistant genotypes has paralleled the increased synthesis of chlorogenic acid, which restricted spread of the pathogen through lignification.

Studies with differential races of *P. infestance* in potato by Henderson and Friend (1979) revealed that induction of PAL is race specific. Avirulent race of *P. infestance* when inoculated either in the form of spores or mycelia to the resistant potato tuber discs enhanced PAL activity. However, fungal extracts failed to activate the enzyme. It was also reported that incompatible interaction also showed deposition of lignin like substances.

Incompatible but not the compatible interaction in tomato and fungus *Verticillium alboatrum* enhanced PAL activity (Grezelinska and Sierakowska, 1975). However, the pre-infection of susceptible plants with a weakly virulent strain as well as γ -rays irradiation induced acquired resistance in plants, thus a later infection by virulent strain showed similar increase in PAL activity as that of a resistant plant. Bernards and Ellis (1991) reported 12-fold increase in PAL activity in tomato cell cultures in response to *Verticillium alboatrum* infection.

In tobacco cultivar NC 2326 resistant to a race 0 of the black shank pathogen *Phytophthora nicotianae* var. *nicotianae*, phenylalanine ammonia- lyase activity got activated rapidly on stem inoculation of the pathogen. Such reaction was not observed in cultivar Hicks, a near isogenic susceptible variety. Aminohydrazinophenyl propionic acid, a specific inhibitor of PAL activity however did not affect the relative susceptibility of either cultivar. But by the incorporation of the systemic phosphonate plant protectant, fosetyl-Al protected the susceptible cultivar Hicks from infection. It enhanced the PAL activity as well (Nemestothy and Guest, 1990). These results bring out the fact that failure to induce PAL results in susceptibility

Stimulation of phenylalanine ammonia lyase activity in tobacco leaves in response to hypersensitivity to tobacco mosaic virus (Fritig *et al*., 1973) emerges as supportive evidence to the earlier findings, showing changes in metabolism of phenolic compounds during hypersensitive reaction in tobacco (Fritig *et al*., 1972).

Studies of Legrand *et al*. (1976) clearly indicated the pronounced increase in PAL activity along with cinnamic acid-4-hydroxylase as an initial response to TMV in tobacco

cultivar Samsun NN (resistant). Increase in enzyme activity was very sharp reaching the maximum at day 2 after TMV inoculation. But the decrease in activity was a gradual process in the subsequent days. The data showed that there is a clear parallel in time and amplitude between necrosis and the stimulation of the phenylpropanoid pathway. Moreover, the total stimulation of enzyme increases with the size of the necrotic area, suggesting PAL as a good biochemical marker of necrotic reaction. Activation of phenyl propanoid metabolism is believed to result in phenolic polymer deposition, which builds up new mechanical barriers against pathogen invasion (Favali *et al*., 1978; Legrand, 1983).

Duchesne *et al*. (1977) from density labeling studies showed evidence for *de novo* synthesis of phenylalanine ammonia-lyase in resistant variety showing hypersensitive resistant reaction on tobacco mosaic virus infection.

Brederode *et al.* (1991) were successful in obtaining only a partial PAL cDNA from a tobacco DNA library. But they did not detect any PAL gene expression during the hypersensitive reaction of tobacco to TMV. Pellegrini *et al*. (1994) isolated a near-full-length cDNA encoding tobacco PAL. Southern blot analysis disclosed the presence of a small family of two to four PAL genes in tobacco. The maximum enzyme activity was recorded 45 hours post inoculation with TMV in tobacco. Studies using fungal elicitor megaspermin, inducing necrosis of tobacco indicated a transient, rapid and sharp induction of PAL transcript accumulation. An increase in enzymatic activity was measured over the same period, in agreement with the fact that PAL messenger quantity remains above the control level. In carborandum wounded samples PAL transcripts accumulated much less than in TMV-infected or elicited tissues. Moreover, 45 hours after

wounding PAL m RNAs dropped to values close to those measured in untreated leaves. Though differences in PAL activity were observed with time of sample collection, these fluctuations were limited compared to the level reached after TMV infection or elicitation. No cell – specific or tissue- specific expression of PAL genes was observed in either the healthy or the infected tobacco leaves.

2.5. Ribonuclease

The hydrolytic breakdown of ribonucleic acid (RNA), in addition to the regulation of RNA synthesis at the transcriptional level plays a major role in the selection of needed molecular species of RNA by the cell at a given moment of its development. The RNA – splitting enzymes belong to the large group of hydrolase enzymes that catalyze the hydrolytic cleavage of C-O, C-N and C-C bonds (Enzyme commission code number: 3). The actual linkages split by the nucleolytic enzymes are ester bonds. The RNA splitting enzymes are, therefore, esterases (EC code: 3.1).

Ribonucleases (RNases) (E.C.3.1.4.22) are widely distributed in both the plant and animal kingdom. The ribonucleases obtained from different sources were shown to differ in the extent of degrading ribonucleic acid. The ribonucleases occurring in bean and tobacco leaves were shown to bring about complete degradation of ribonucleic acid (Holden and Pirie, 1955a; Markham and Strominger, 1956; Frisch-Niggemeyer and Reddi, 1957; Reddi, 1958).

Ribonucleases have been isolated from a large variety of plants including tobacco (Jervis, 1974). Reddi and Mauser (1965) and Jervis (1974) have reported a particle – bound RNase, similar to corn RNase II from tobacco.

Plants exhibit changes in RNase levels in response to a variety of different endogenous and exogenous stimuli. They are senescence, phosphate starvation, plant disease, light and other developmental signals as well (Green, 1994). Ribonucleases are likely to play important functional roles in plants such as self-incompatibility, phosphate remobilization, plant defense, and mRNA decay (Green, 1994).

Steady increase in RNase activity of leaf during the process of senescence was reported by La'za'r and Farkas (1970). The deteriorative processes associated with senescence seem to trigger the increase in RNase activity. Works of Dove (1971), Pitt and Galpin (1971) in leaflets of tomato and leaf as well as tuber tissue of *Solanum tuberosum* respectively have indicated biphasic nature of enzyme activity, with a rapid increase early after isolation, followed by, a slight decrease and a second increase in enzyme level. The first phase of increase was attributed as a response to wound and the second to senescence or death of plant part.

Wounding (Rubbing or cutting) of leaf in tobacco and *Solanum tuberosum* induced rapid increase in RNA-splitting enzyme activity, in some cases within 1 to 2 hours after treatment (Bagi and Farkas, 1967; Pitt and Galpin, 1971). The short-term response of RNase activity to leaf detachment was reported for several plant species (Dove, 1973). It is apparently due to mechanical injury. This phase of increase in RNase level does not seem to be an early "Biochemical Marker" of the onset of senescence. The pattern of nucleolytic enzymes once established in the damaged tissues, remains unchanged for a considerable time and does not resemble the typical nucleolytic enzyme spectrum of the ageing leaves.

The extent of the increase in RNase activity in excised tobacco leaf discs is correlated with number of cells actually damaged. The stimulus is translocated from the directly affected cells to zones not directly affected (Bagi and Farkas, 1967). They have also showed that actinomycin D treatment inhibits rise in RNase activity.

On the basis of immunochemical and incorporation studies including preliminary experiments by the use of density labeling, Pitt (1971, 1974, 1975) concluded that only a small portion of RNase, especially lysosomal RNase is synthesized *de novo* in damaged potato leaves and tuber.

No sound hypothesis could be arrived at as regards the physiological significance of wound-RNase production. Pitt (1975) is of the opinion that the enzymes probably soon after their synthesis, become secluded in the lysosome, thus separates effectively the enzymes and their substrates. In a later phase, due to autophagy and total cell collapse, the RNA-degrading enzymes may digest a major part of cellular RNA (Matile, 1978).

Farkas (1978) opined that there is no evident reason why the plant needs large reservoir of hydrolytic enzyme, except for the increase of lysosomal enzymes during aging and probably during disease. Perhaps in plant disease and senescence, lysosomes temporarily play a protective role by compensating for a regulatory system that went wrong because of a defect in disease and / or senescence and over produced hydrolytic enzymes.

Works of Chakravorty *et al*. (1974 a&b) as well as Chakravorty and Scott (1979 a&b) on wheat and flax rust diseases, have prompted the postulation of parasitically induced synthesis of specific RNA-splitting enzymes. It was also proposed

that the changes in nucleolytic enzyme activity have a bearing on the altered RNA transcription patterns in the diseased plant tissue. Most of the wok in this direction suggests the formation of specific enzymes upon infection possibly important for the outcome of host parasite relationship, has been done on tissues attacked by rusts and powdery mildew. These pathogens elicit a biphasic increase in RNase activity in the infected tissues. The second peak is induced only in susceptible host parasite combinations. The separation of host and parasite functions is not easy in host parasite systems, but the results suggest that nucleolytic enzymes in the infected tissues differ in some of their properties from those found in the corresponding non-infected plants. The intensive RNA synthesis and accumulation in and close to the infection centers were reported. Part of this is assigned to the contribution of the cells of the parasite, but a wounding effect, inducing increased RNA synthesis in the host cells is also important.

At a certain distance from the infection centers, the leaf tissues often turn yellow as an indication of premature senescence. In these areas of the leaf, the chloroplasts show early signs of deterioration. Infection with necrotrophic parasites, especially with pathogens producing toxins, leads to premature yellowing of the leaves. RNA break down has been observed in such systems (Lovrekovich *et al*., 1964). Works on virus – infected tobacco leaves by Reddi (1959) paved way for accumulation of evidence showing higher RNase activity in a number of diseased plant tissues than in the comparable controls (Dove, 1973; Wilson, 1975).

Identification of ribonuclease in tobacco by Pirie (1950) followed by its partial purification by Holden and Pirie in 1955 has led to the intensive work on this ribonuclease in later years. Frisch-Niggemeyer and Reddi in 1957 have refined the

method to achieve 100-fold purification. They also found that the pH optimum of this enzyme is 5.1. Studies on mechanism of action of tobacco leaf ribonuclease by Reddi (1958) indicated that this enzyme cleaves all the inter nucleotide linkages in ribonucleic acid giving rise to nucleoside 2': 3'-cyclic phosphates. The enzyme had no action on pyrimidine cyclic nucleotides while it slowly hydrolysed purine cyclic nucleotides to nucleoside 3'- phosphates exclusively.

Reddi (1959) correlated the TMV synthesis with RNase activity in the leaf and suggested role of RNase in the synthetic process leading to the formation of viral nucleic acid. Since, TMV does not possess any known independent enzyme activities, RNase is rapidly formed in locations of the plant where such activities are needed for its synthesis. He also showed that the level of RNase activity varied with leaf age, bottom leaves contained more RNase than the top young leaves. In case of Turkish tobacco plants in which TMV multiplies systemically (susceptible), more virus particles were found in the bottom leaves than in the top leaves. Thus, the extent of TMV formation appears to parallel the RNase activity of the leaves.

The role of RNase in the infected leaves possibly is to break down leaf RNA to facilitate the synthesis of viral RNA by a process of rearrangement of break down products.

Studies on Turkish tobacco by Reddi (1963) showed rapid degradation of microsomal RNA in leaves infected with TMV and utilization of the degradation products in the synthesis of TMV-RNA as evidenced by the increased level of TMV-RNA. Because the activity of RNase increased following infection with TMV it was

proposed that the leaf RNase might degrade microsomal RNA in the infected tobacco leaf. Evidence for the utilization of the breakdown products of the microsomal RNA in the synthesis of TMV-RNA was provided by tritiated uridine labeling studies. From the studies, it was found that the microsomal RNA contributed about 68 percent of the uridylic acid of TMV-RNA and the rest probably is derived by *de novo* synthesis. It was concluded from the studies that microsomal RNA, which forms about 55 to 60 percent of the total leaf RNA, acts as a reservoir supplying the ribonucleosides for the synthesis of TMV-RNA. This supported the earlier hypothesis of Reddi (1959) correlating RNase activity and virus synthesis.

Reddi and Mauser (1965) have isolated leaf RNase, which has pH optimum of 5.8 to 6.0 and found it loosely bound to the microsomes. This was identified as tobacco leaf ribonuclease-II (TLRNase II), and was different from the tobacco leaf ribonuclease-I (TLRNase I) identified earlier having pH optimum of 5.1. The TLRNase II was capable of hydrolysing both purine and pyrimidine 2', 3'-cyclic nucleotides to their corresponding nucleoside 3' – phosphates. Based on these experiments the possible mode of degradation of cellular RNA to ribonucleosides and the pathway for the biosynthesis of the TMV-RNA in which ribonucleosides derived from the host RNA serving as intermediates was proposed as follows.

$$\text{Host RNA} \xrightarrow[\text{TLRNase II}]{\text{TLRNase I}} \text{Ribonucleoside 3'-phosphates}$$

$$\text{Ribonucleoside 3'-phosphates} \xrightarrow{\text{Phosphatase}} \text{Ribonucleosides + inorganic Phosphate}$$

$$\text{Ribonucleosides + ATP} \xrightarrow{\text{Nucleoside phosphokinase}} \text{Ribonucleoside 5'-phosphates +ADP}$$

Jervis (1974) has isolated and partially purified two enzymes with similar properties that degrade RNA but not DNA in tobacco leaves. They were termed RNase I and RNase II having molecular weights of 19,700 and 21,000 respectively. The former had pH optima of 5.1 and the latter 5.8 to 6.0. Their distribution was at a ratio of 1:1 in young leaves and up to 5:1 in older leaves.

In a tobacco/TMV local lesion combination, the early increase in RNase activity was found to be due to an increase in the amount of the relatively guanine-specific endoribonuclease, the activity of which also increased upon wounding the tissue (Wyen *et al.,* 1972).

Results of several studies indicated that the increase in RNase activities is more pronounced in compatible rather than incompatible reactions. Recent works of Lee *et al.* (1992) indicated the role of RNase in defending plants against pathogen attack in *Petunia* and *Arabidopsis*. This led Green (1994) to suggest a new hypothesis towards identifying R Nases having anti-viral, anti-bacterial or anti-fungal activity (degradation activity) and engineer pathogen-resistant plants that secrete such RNases in large amounts.

Sindelarova *et al*. (2002) conducted a detailed study of changes in enzyme including ribonuclease that control host rRNA degradation in tobacco leaves. They observed a sharp increase in the rRNA degrading enzymes during the acute phase of TMV multiplication in infected susceptible cultivar Samsun and the curves of these activities were positively correlated with the multiplication curves of TMV.

2.6. Salicylic Acid

Salicylic acid or *O*-hydroxyl benzoic acid belongs to the group of plant phenolics that possess an aromatic ring bearing a hydroxyl group or its functional derivative. Salicylic acid gained importance since 1874 because of its therapeutic property when acetyl salicylic acid was produced commercially and given the name Aspirin, though its use has been seen since 4th century BC as an extract for curing aches and fevers.

Plant phenolic compounds (earlier classified as secondary metabolites of minor importance) slowly gained importance and were implicated to play an essential role in regulation of plant growth, development, and interaction with other organisms (Harborne, 1980). Experimental evidences showed that, phenolic compounds function as signals in plant-microbe interactions as well, in addition to their role as chemical defenses of plants against microbes, insects and herbivores (Metraux and Raskin, 1992). Phenolics act as allelopathic compounds influencing germination and growth of neighbouring plants (Einhellig, 1986) and in the activation of parasitic *Striga* species to enable its attachment to host root (Lynn and Chang, 1990). A comprehensive survey of salicylic acid in leaves and reproductive organs of several angiosperms confirmed the ubiquitous distribution of this compound in plants (Raskin *et al.,* 1990).

van Herk (1937 a&b) proposed the hypothesis that calorigen is associated with heat production in thermogenic plants. This lead to the identification of calorigen to be salicylic acid (Raskin *et al.,* 1987). Stimulatory effects of salicylic acid in flowering were later demonstrated in Lemnaceae (Cleland and Ajami, 1974; Khurana and Maheshwari, 1980). Accumulation of salicylic acid following initial infection with necrotizing

pathogens was demonstrated in Cucumber, Tobacco and *Arabidopsis* (Malamy *et al.,* 1990; Metraux *et al.,* 1990); Rasmussen *et al.,* 1991; Uknes, *et al.,* 1993).

Salicylic acid has been implicated as one of the key components in the signal transduction pathway, leading to plant resistance to various pathogens (Ryals *et al.,* 1996; Wobbe and Klessig, 1996). Although, there is still controversy about the nature of long distance signal (Shulaev *et al.,* 1995; Vernooij *et al.,* 1994), it is evident that salicylic acid is involved in the signal transduction. It is required for the establishment of non-localized and long lasting induced protection called systemic acquired resistance (SAR) (Ross, 1961), which prevent infection of a broad spectrum of pathogens. Since then several reviews have been published regarding systemic acquired resistance and plant signal transduction (Raskin, 1992; Yalpani and Raskin, 1993; Ryals *et al.,* 1994; Bowler and Chua, 1994; Baratali and Farshad, 2015).

Resistant but not the susceptible cultivars produce several new proteins in response to Tobacco Mosaic Virus (TMV) infection, which include five distinct families of proteins referred to as pathogenesis related (PR) proteins. Association of pathogenesis related genes with resistance suggested the involvement of these proteins in systemic acquired resistance (Carr *et al.,* 1985). Application of exogenous salicylic acid or its derivative acetyl salicylic acid was shown to be effective in triggering pathogenesis related genes to produce PR proteins in many plants including tobacco (White, 1979; White *et al.,* 1987) thereby suggesting salicylic acid formation is a prerequisite for induction of PR proteins.

Application of salicylic acid resulted in increased resistance of the treated areas to tobacco mosaic virus and some other viruses (von Huijsduijnen *et al.,* 1986). The

movement of salicylic acid from TMV-inoculated leaves of Xanthi-nc tobacco and its presence in phloem tissue was confirmed using excised leaf technique developed by Malamy *et al.* (1990). Experimentation on tobacco cultivars Xanthi (TMV susceptible) and Xanthi-nc (TMV resistant) showed that salicylic acid concentration begins to increase during 24 to 36 hours after inoculation of TMV. The increase is 20-fold or more over basal levels by 42 to 48 hours in TMV infected leaves of Xanthi-nc cultivars. Elevated level noticed in the infected leaves triggered increase in salicylic acid levels in other leaves of the same plant (systemic increase). The systemic increase of salicylic acid in uninfected leaves of the same plant, was followed by the appearance of pathogenesis related protein (PR1) m RNA' s, which was detected at 72 hours after inoculation and increased in quantity thereafter. In susceptible cultivars Xanthi, the PR genes could be activated by treatment with exogenous salicylic acid, but not with TMV inoculation. This shows that infection of susceptible plants fails to trigger the signal transduction pathway that leads to salicylic acid production, PR gene expression and. resistance

Similar studies conducted by Metraux *et al.* (1990) in cucumber showed that salicylic acid plays an important role in the induction of systemic acquired resistance on pathogen attack.

Findings of Yalpani *et al.* (1991) also supported the view that salicylic acid accumulation in tobacco leaf tissue and stele tissue is an integral part of the hypersensitive reaction and is sufficient for the induction of pathogenesis related proteins, commonly observed during systemic acquired resistance. The temperature effects on the hypersensitive reaction and salicylic acid accumulation were clearly elucidated by their experimentation. At 32ºC salicylic acid accumulation is inhibited and it is also

accompanied by the disappearance of hypersensitive reaction and PR protein, thus providing the interdependence of the three components in tobacco. van Loon (1975) demonstrated inhibition of both hypersensitive reaction and PR-1 protein accumulation in TMV-inoculated Xanthi-nc on incubating the plants at 32ºC. Induction of salicylic acid and its conjugates during resistance response to tobacco in relation to temperature shift was studied by Malamy *et al*. (1992). When Xanthi-nc plants kept at 32ºC were inoculated with TMV, salicylic acid level did not increase. Whereas, salicylic acid level in control plants inoculated and maintained at 22ºC, rose by 10 to 20-fold. When plants inoculated with TMV at 32ºC were shifted to 22ºC, resistance was established and salicylic acid level rose ten folds over basal levels by 6 hours post shift and 100 to 200 folds above basal level by 10 hours post shift. Estimation of level of conjugated salicylic acid during the post infection period at regular intervals showed dramatic increase of conjugates between 24 and 48 hours post inoculation at a level two times greater than the amount of salicylic acid. The ability of β - glucosidase to hydrolyze the vast majority of salicylic acid conjugates in TMV infected leaves, strongly suggested the involvement of salicylic acid glucosides as the major form of salicylic acid conjugates. It may serve as a storage pool of SA.

The possible production of salicylic acid in the plants as a sugar conjugate was proposed by Malamy *et al.* (1990). When leaf extract from TMV – inoculated Xanthi-nc tobacco cultivar was treated with β-glucosidase, salicylic acid yield increased by 33-fold. The majority of salicylic acid found in the leaf 3 days after TMV inoculation was found in conjugated form (Enyedi *et al*., 1992). Salicylic acid inducible glucosyl transferases, which conjugate free salicylic acid with UDP-glucose to form O-β-D-glucosyl-salicylic

acid has been isolated from oat roots by Balke *et al*. (1987). Hence, Enyedi and co-workers tentatively identified this conjugate as O-β-D-glucosyl-salicylic acid. It was also indicated that free salicylic acid rather than the conjugate exists in the phloem, indicating salicylic acid as a better mobile form than glucosyl-salicylic acid. No detectable quantity of salicylic acid glucoside was noticed in upper uninoculated leaves even at 48 to 144 hours after TMV inoculation. A steep increase in salicylic acid concentration gradient was established in leaf tissue surrounding individual lesions. Salicylic acid level rapidly declined away from the center of the lesion to the extent of nearly 27-fold lower at 6.5 to 10 mm. distance.

Enyedi and Raskin (1993) identified β-glucosidase hydrolyzable salicylic acid conjugate as β-O-D-glucosyl salicylic acid. Salicylic acid and β-O-D-glucosyl salicylic acid accumulation in the TMV inoculated leaf paralleled the increase in the activity of a UDP-glucose. Salicylic acid 3-O-glucosyl transferase (EC 2.4.1.35) is capable of converting salicylic acid to β-O-D-glucosyl salicylic acid. Activity of this enzyme started to increase 48 hours after TMV inoculation and reached its maximum at 72 hours. Salicylic acid levels present in the inoculated leaves were sufficient for the induction of this enzyme.

β-glucosyl transferase induction was specific for salicylic acid and its close analogue 2,6-dihydroxy benzoic acid. The ability of SA to induce β-glucosyl transferase activity may serve as an effective feedback regulation mechanism for the maintenance of SA level in plant tissues. This may serve as a key factor in the signal transduction pathway by activation of disease resistance mechanism. Hence, β-glucosyl transferase enzyme is proposed to be referred to as a pathogenesis-related protein.

Plants most likely produce salicylic acid (O-hydroxybenzoic acid) due to the activity of phenylalanine ammonia-lyase on phenylalanine. This enzyme is induced by a range of biotic and abiotic stresses and is a key regulator of the phenyl propanoid pathway (Fig. 2.6.1), which yields a variety of phenolics with defense related functions (Dixon and Paiva, 1995).

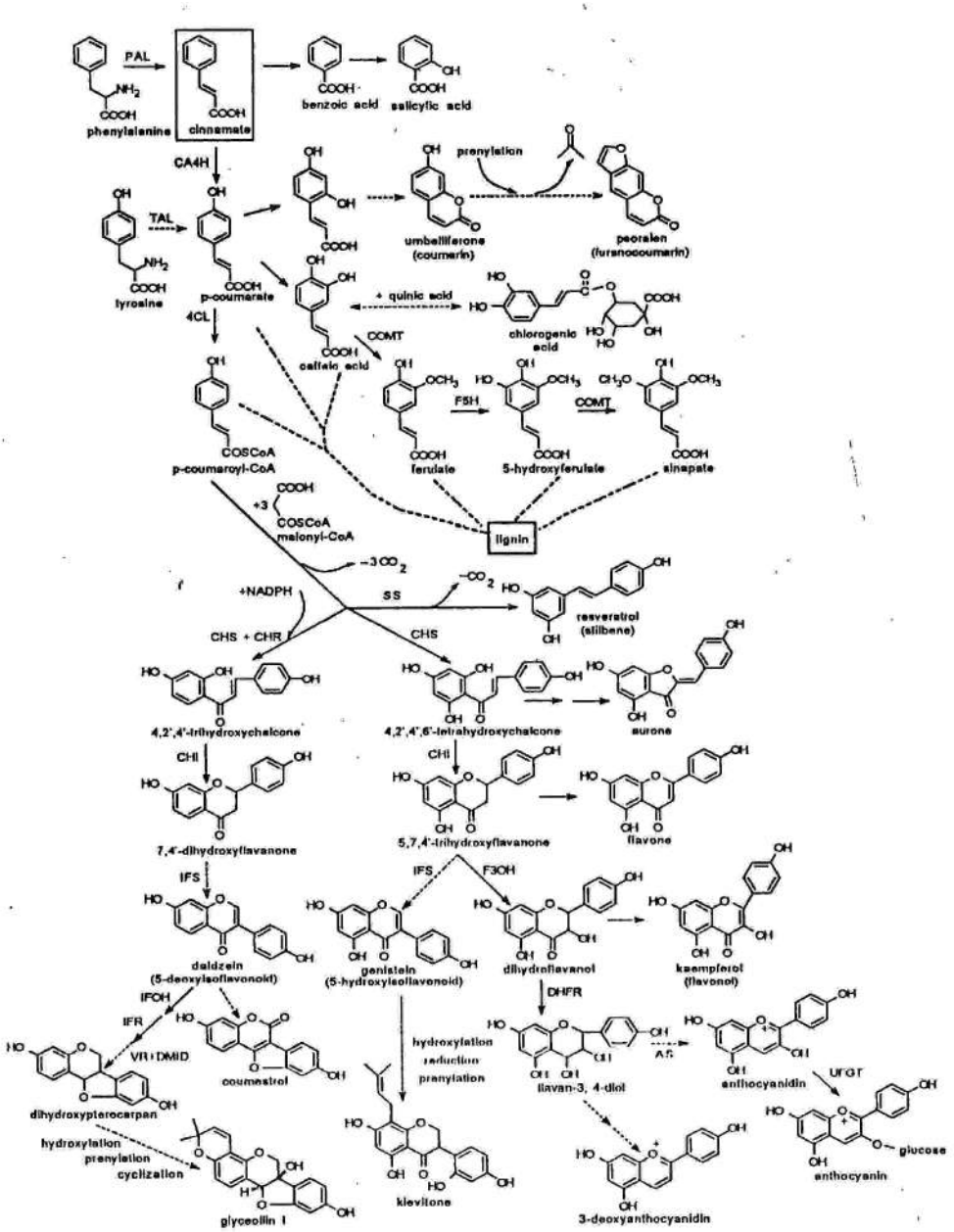


Fig. 2.6.1. Biosynthetic relationships among stress-induced phenylpropanoids (Dixon and Paiva, 1995)

Two pathways for the formation of salicylic acid from cinnamic acid have been known in plants as detailed in Fig. 2.6.2.

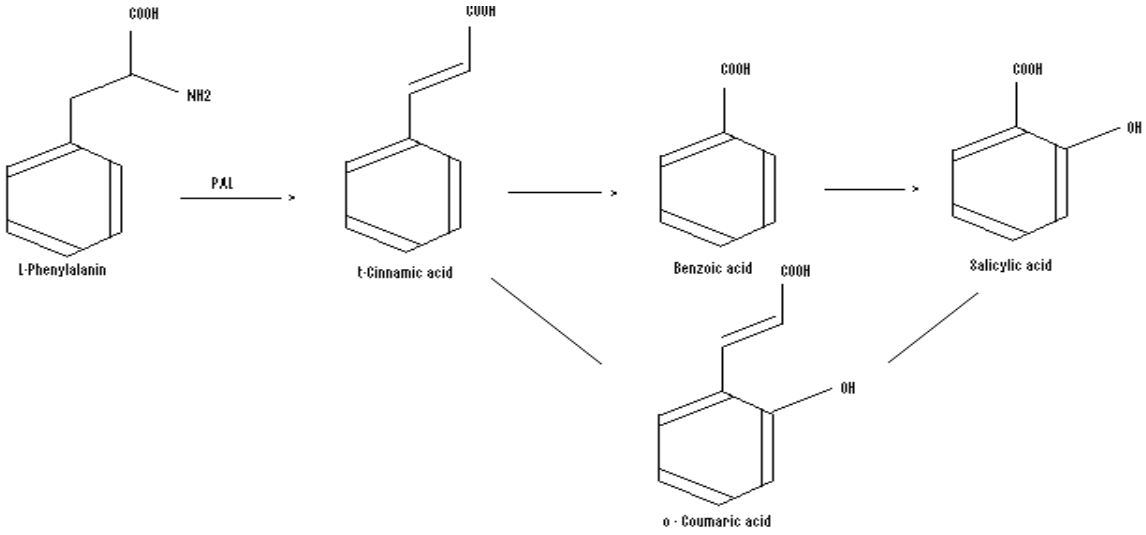


Fig. 2.6.2. Pathway of salicylic acid biosynthesis in plants

The works of Yalpani *et al*. (1993 a) indicated that both in healthy and TMV inoculated tobacco salicylic acid is formed from cinnamic acid via. benzoic acid. In healthy Xanthi-nc tobacco leaves, cinnamic, O-Coumaric and benzoic acid are present in conjugated forms of these compounds. In TMV inoculated tissue, the conjugated form of benzoic acid transiently decreased resulting in accumulation of free benzoic acid due to the hydrolysis of conjugated benzoic acid indicating the need for benzoic acid probably to make salicylate. The feeding experiment showed that salicylic acid content increased only when benzoic acid is fed. These results support the view that most if not all salicylic acid is formed form cinnamic acid viz. benzoic acid and the levels of benzoic acid influence salicylic acid accumulation in tobacco and these changes are attributed to TMV infection.

Radio labeling studies of Meuwly *et al*. (1995), showed that salicylic acid, an essential component in the signal transduction pathway leading to systemic acquired resistance in cucumber plants inoculated with pathogens is synthesized from phenylalanine and benzoic acid. This further supports the view of the benzoic acid pathway in the formation of salicylic acid.

Pathogen induced regulation of salicylic acid

Detection of benzoic acid 2-hydroxylase in healthy tobacco leaf extracts and recording significant increase of this enzyme upon inoculation with TMV further supports the operation of benzoic acid pathway hypothesis in salicylic acid formation in plants (Leon *et al*., 1993). The increase in enzyme level coincided with higher concentration of free salicylic acid in the leaves. The magnitude and timing of the benzoic acid increase observed in TMV inoculated plants as well as the induction of benzoic acid 2-hydroxylase by exogenous application of benzoic acid implies that an increase in the benzoic acid pool is required for benzoic acid 2-hydroxylase induction. Feeding tobacco leaves with phenylalanine, cinnamic acid, or *O*-coumaric acid failed to trigger the induction of benzoic acid 2-hydroxylase activity. Benzoic acid 2-hydroxylase appears to be a pathogen-inducible protein with an important regulatory role in salicylic acid accumulation during the development of induced resistance to TMV.

Leon *et al*. (1995 a) isolated and characterized the high molecular weight, soluble P_{450} oxygenase, benzoic acid 2-hydroxylase from tobacco. The isolation of benzoic acid 2-hydroxylase genes from plant may provide a powerful tool to manipulate salicylic acid levels in plants, thereby altering the resistance to the pathogens.

Yalpani *et al. (*1993 b) put forth the theory that crops with elevated levels of endogenous salicylic acid should have greater resistance to pathogen, based on their studies with *N. glutinosa* x N. *debney* an amphidiploid hybrid of resistant *N. glutinosa* ('N' genotype) and the susceptible *N. debney* ('n' genotype). This hybrid exhibits a higher degree of resistance to TMV infection than either of its parental species or Xanthi-nc tobacco (Ahl and Gianinazzi, 1982). Salicylic acid in healthy leaves of hybrid, constitutively expresses PR proteins, 30 times higher than the salicylic acid levels in the young unchallenged leaves of Xanthi – nc tobacco, which show lower resistance to TMV. The amount of salicylic acid present in the leaves of a healthy hybrid is much greater than the minimum level needed for the induction of PR proteins and TMV-resistance in Xanthi-nc tobacco. No qualitative or quantitative changes in the acidic proteins were induced by treating the hybrid with TMV or salicylic acid suggesting that the induction of PR proteins by salicylic acid is close to saturation in this genotype. Alternatively, non-increase of salicylic acid in high proportions on TMV inoculation in hybrid may be explained by the fact that higher basal level of salicylic acid and 80 times smaller necrotic lesions in hybrid than that in Xanthi-nc tobacco.

Similar to the effect of TMV inoculation irradiation with UV light or exposure to ozone, that generates oxygen radicals and H_2O_2, were shown to induce benzoic acid 2-hydroxylase, and accumulate salicylic acid in tobacco plants (Yalpani *et al.,* 1994). The response to H_2O_2 in tobacco leaves and effects of peroxides on benzoic acid, 2-hydroxylase were studied by Leon *et al*. (1995 b). Application of H_2O_2 to tobacco leaves at concentration below the phytotoxicity threshold induced a dose dependent accumulation of salicylic acid and benzoic acid. In addition, H_2O_2 induced benzoic acid

2-hydroxylase, which catalyzed the conversion of benzoic acid to salicylic acid *in vivo* and *in vitro*. The oxidative burst, which follows the pathogen recognition, results in production of active oxygen species and H_2O_2 in and around the infection site. The effects of these molecular species contribute to rapid cell death during the hypersensitive reaction (Levine *et al*., 1994). Based on the data and literature Leon *et al*. (1995b) proposed a model of the biochemical events leading to acquired resistance (Fig. 2.6.3).

Gaffney *et al*. (1993) used transgenic tobacco plants harbouring nah G gene that codes for salicylate hydroxylase (E.C. 1.14.13.1) enzyme catalyzing decarboxylative hydroxylation of salicylate, to catechol. The results showed that, expression of nah G mRNA and salicylate hydroxylase in transgenic plants significantly blocked salicylic acid accumulation in TMV treated plants. These transgenic plants were defective in their ability to induce acquired resistance against tobacco mosaic virus. These findings proved that salicylic acid accumulation is required for the systemic acquired resistance. This has become the most compelling evidence that salicylic acid has a role in the signal transduction pathway leading to systemic acquired resistance.

Though salicylic acid has been implicated as a component of signaling pathway, very little is known about the pathway as such, which leads to the systemic resistance state following pathogen infection. Salicylic acid has not been proved as a long distance, phloem – transmissible signal to move from the site of initial pathogen infection to all parts of the plant. Vernooij *et al*. (1994) investigated whether salicylic acid could be the systemic signal by taking advantage of the observations that the translocating signal can pass through a graft junction (Jenns and Kuc, 1979; Gianinazzi and Ahl, 1983).

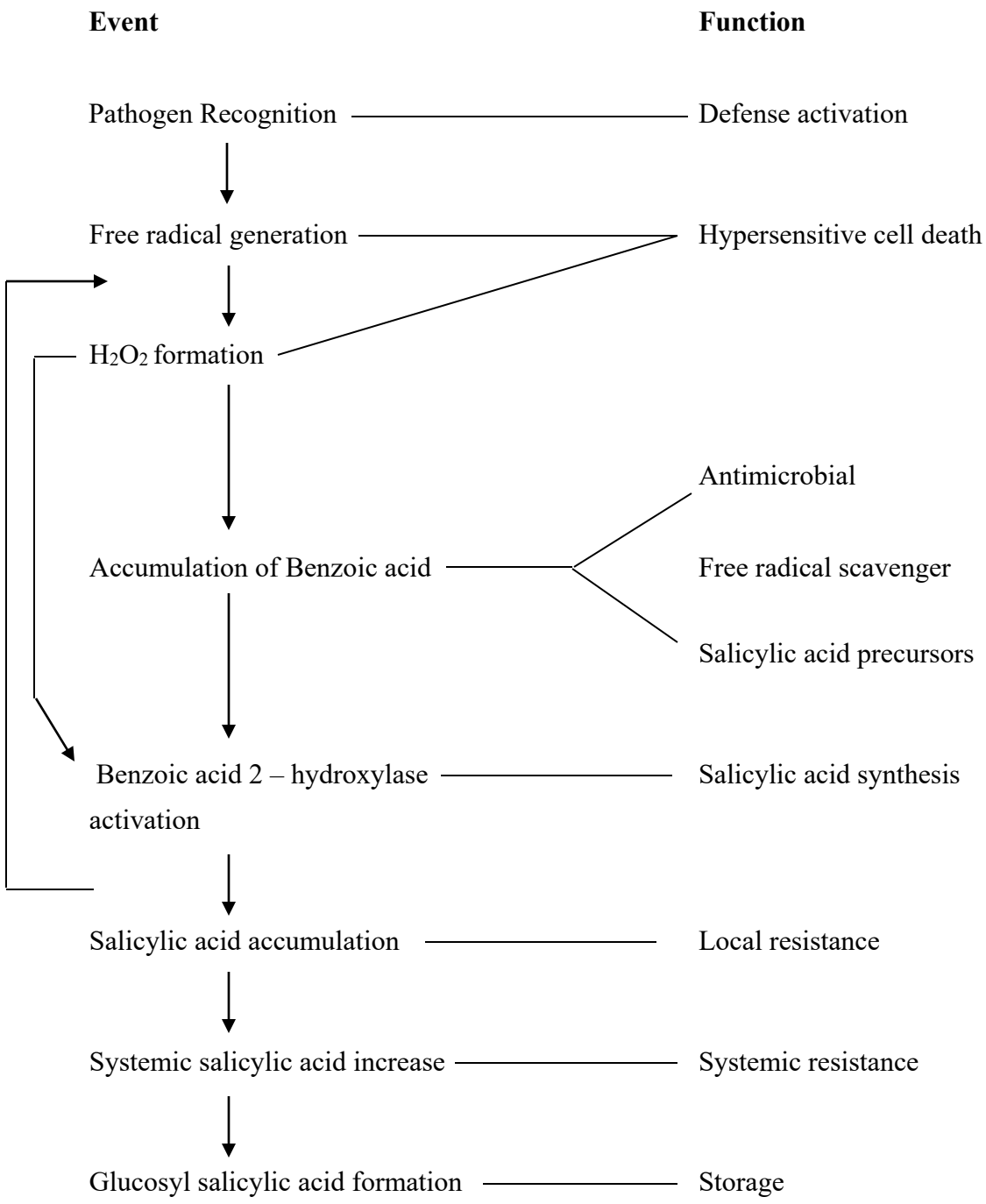


Fig. 2.6.3. Pathogen Defense Pathway

Despite their inability to accumulate salicylic acid, tobacco mosaic virus-infected root-stocks of transgenic Nah G plants were capable of inducing resistance to viral and fungal pathogens in grafted wild type tissues, indicating that salicylic acid is not the

systemic signal. In contrast, wild type and Nah G rootstocks were not able to induce resistance in Nah G scions, demonstrating that the long-distance signal requires the presence of salicylic acid to induce the resistance state in systemic tissues. These results lead to the conclusion that in a model of systemic acquired resistance, pathogen induced local necrosis leads to production of an unidentified vascular mobile factor. However, presence of salicylic acid in distal tissues is needed for the establishment of the resistant state.

To resolve the controversy whether salicylic acid is or is not the systemic signal for systemic acquired resistance, Chasan (1995) suggested to repeat the grafting experiments using a mutant that could not synthesize salicylic acid rather than one that degrades it once it is formed. If salicylic acid is really the systemic acquired resistance signal, then biosynthetic mutant scion leaves should respond to salicylic acid translocated from a wild type rootstock. Conversely of a null biosynthetic mutant root stock sends a signal that causes systemic acquired resistance to be established in wild type scion leaves, this would demonstrate conclusively that salicylic acid is not the signal for systemic acquired resistance.

The studies by Keller *et al*. (1996) indicated that accumulation of salicylic acid is a pre-requisite for the establishment of elicitin induced systemic acquired resistance, but is not required for necrosis. Analyses of m RNA accumulation revealed that the expression of a sub set of elicitin responsive systemic acquired resistance genes is suppressed in Nah G tobacco. In contrast transcripts from other systemic acquired resistance genes and from the so-called **'str'** genes accumulate regardless of whether this Nah G gene is expressed or not. The results demonstrated that salicylic acid is an

essential intermediate in the establishment and maintenance of elicitin induced systemic acquired resistance and an important factor for disease resistance to *Phytophthora* but salicylic acid seems not to be the general mediator of hypersensitive reaction.

He Du and Klessig (1997) showed that salicylic acid is also necessary for the induction of systemic acquired resistance and to certain defense responses such as PR – 1 gene expression to tobacco mosaic virus in catalase deficient plants. However, salicylic acid does not appear to be required for the development of necrosis, which is likely to be brought about by reduction of catalase activity indicating that necrotic lesion formation can be uncoupled from disease resistance. Hence, there seems to be a salicylic acid independent pathway controlling the formation of necrosis, in addition to the salicylic acid dependent pathway that leads to the induction of PR genes and enhanced resistance.

Although, much experimental data accumulated over the years support the role of salicylic acid as a primary long distance signal in systemic acquired resistance, several results suggest that salicylic acid may not be the translocated signal responsible for the induction of systemic acquired resistance. Study of Shulaev *et al.* (1995) in tobacco mosaic virus resistant Xanthi-nc tobacco, showed that salicylic acid increases systemically following the inoculation of a single leaf with TMV. Non-invasive, *in vivo* labeling of salicylic acid produced in TMV inoculated or uninoculated leaves with oxygen – 18 showed magnitude and timing in distribution of $^{18}0^{-}$ salicylic acid, thus indicating that most salicylic acid detected in the healthy tissues are synthesized in the inoculated leaf. Systemic movement of salicylic acid in the tobacco plant was also confirmed by the detections of ^{14} C labeled salicylic acid in the upper leaves when it was injected into lower leaves. Data indicated that a substantial part (60 to 70%) of salicylic

acid accumulated in the upper leaves arrived from the inoculated lower leaf. The rest is hypothesized to be produced from benzoic acid, which is also exported into upper leaves with salicylic acid. Detection of highest systemic increase in salicylic acid in the youngest leaves located directly above the inoculated leaf further supports the translocation of salicylic acid through phloem. Detection of low level of salicylic acid in upper uninoculated leaves reported in earlier studies is presumed to be because these leaves were located approximately opposite to inoculated leaf and have relatively little direct vascular connection with inoculated leaf. The data indicated that detaching the inoculated leaf before it starts to accumulate salicylic acid, completely blocks systemic salicylic acid increase in upper leaves. Even more importantly PR-1 gene expression and systemic acquired resistance were not detected in the upper leaf unless lower leaves were allowed to accumulate salicylic acid before its detachment from the plant. On giving explanations to the earlier findings not supporting the salicylic acid tansduction, it is concluded that at least in the upper leaves of TMV inoculated Xanthi nc tobacco, salicylic acid is an important translocated signal involved in systemic acquired resistance and systemic induction of PR proteins.

Zhu *et al*. (2014) have studied the role of salicylic acid and jasmonic acid in systemic resistance responses in *Nicotiana benthamiana* using virus induced gene-silencing (VIGS) – based genetic approach. The study revealed that methyl salicylate and methyl jasmonates are essential for systemic resistance against tobacco mosaic virus and attributed their role as signal initiators. Silencing the genes involved in signaling and biosynthesis of salicylic acid and jasmonic acid in *N. benthemiana* plants increased susceptibility, and thus demonstrated their need for systemic resistance response against TMV.

3. Materials and Methods

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Collection of seeds of different genotypes used in the present study

Seeds of *Nicotiana tabacum* cultivars Va 770, Vamorr 48, Vamorr 50, Vamfen Hicks, TMVRR 3, TMVRR 2, Beinhart 1000-1, Riwaka 1, 2640-4, 2663-5, 2687-7, 2693-3, and 2700-6 having resistance to tobacco mosaic virus (TMV) disease, FCH 6248 having tolerance to TMV, FCV Special, Bhavya and Swarna susceptible to TMV and wild species *Nicotiana glutinosa* were obtained from CTRI Research Station, Hunsur. Viable seed stocks were regenerated from the cultivars grown in pots.

3.1.2. Chemicals

Coomassie Brilliant Blue G-250, O-Dianisidine hydrochloride, Salicylic acid, Linoleic acid, Catechol, *L*-Phenylealanine, t-Cinnamic acid, β-mercapto ethanol, Silica gel, Ribonucleic acid, Bovine serum albumin, Polyethelene glycol, Carborundum 200-400 mesh, Acrylamide, Bisacrylamide, Ammonium persulphate, TMED and all other common chemicals and solvents were of analytical grade, obtained from reputed fine chemical manufacturers.

3.2. Plant culture and growth conditions

3.2.1. Seedling production in laboratory

The seeds were surface sterilized with a 1% sodium hypochlorite solution for 5 min and washed three times with sterile water to remove surface borne micro – organisms if any. These surface sterilized seeds were spread on moist Whatmann filter paper discs placed inside Petri dishes and incubated in seed germinator at 25±1^{o}C under darkness (for

required number of days). Sterile water was added during the course of germination to maintain the required moisture level.

3.2.2. Seedlings production in shade house

Seeds were sown in polyethylene cups of 6 cm size filled with sterilized sand / acid washed sand. These cups were arranged in trays (Fig. 3.2.1 and 3.2.2) and kept in shade house under ambient conditions viz. 50 to 75% relative humidity, daytime temperature of 26 to 28ºC and night temperature of 16 to 20ºC and natural photoperiods. The cups were irrigated with sterile water till germination (upto 7 days), followed by half-strength Hoagland's solution (Hoagland and Arnon, 1950) for three weeks and then changed to full strength nutrient solution (Table 3.2.1).

Table 3.2.1. Composition of full strength Hoagland's solution

Chemical	**Quantity**	**Micronutrient stock solution**	
1*M* KH_2PO_4	1 mL	**Chemical**	**Quantity (%)**
1*M* KNO_3	5 mL	H_3BO_3	0.286
1*M* $Ca(NO_3)_2$	2 mL	$MnCl_2 . 4H_2O$	0.181
1*M* Micronutrient stock	1 mL	$ZnSO_4 . 7H_2O$	0.022
0.5% Fe tartrate	1 mL	$CuSO_4 . 5H_2O$	0.008
Water	To make 1L	$H_2MoO_4 . H_2O$	0.002

The seedlings aged 15 days into polyethelene cups of 6 cm diameter containing acid washed sand (Fig. 3.2.3 and 3.2.4) and seedlings aged 30 days were transplanted into 23 cm earthen pots of 23 cm diameter filled with sterilized compost and fed with 10 to 50 mL (depending on the container size) half strength Hoagland's solution on alternate days and sterile water was used as needed at all other times.

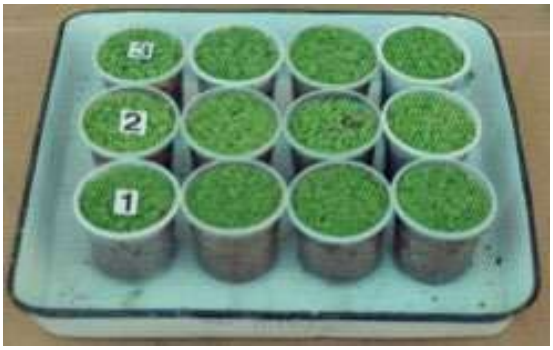


Fig. 3.2.1. Sand culture of tobacco seedlings using polyethylene cups in shade house.

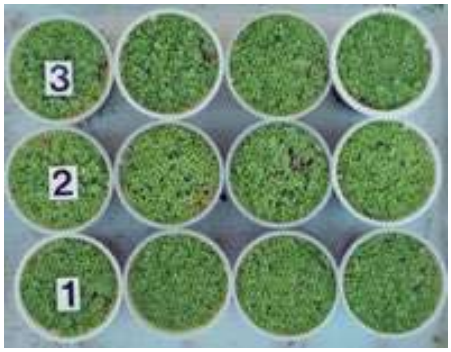


Fig. 3.2.2. A close up of seedlings of three varieties raised through pot culture. (1. FCV Special; 2. FCH 6248; 3. Va 770)

Fig. 3.2.3. Transplanted seedlings in growing cups.

Fig. 3.2.4. A close-up view of transplanted seedlings growing in cups.

3.3. Methods

3.3.1. Peroxidase extraction and estimation

Extraction

0.5 g of the tissue (seed/leaf) samples were taken in triplicate and macerated in a pre-cooled mortar at 4ºC with an equal quantity of acid purified sand and 5.0 mL of chilled 0.05 M sodium phosphate buffer (pH 6.5). The homogenate was passed through a double layer of cheesecloth. The filtrate was centrifuged at 15000 x g for 10 min at 4ºC in a Sorvall RC 5B refrigerated super speed centrifuge. The supernatant was decanted to test tubes kept in ice bath and used as the enzyme source.

Assay

Proxidase assay was performed by using *O*-dianisidine as the proton donor in the presence of H_2O_2 as substrate following the method described by Malik and Singh (1980). The enzyme activity was measured by recording the absorbance at 430 nm using Shimadzu UV-160A automatic recording spectrophotometer.

The reaction mixture (4 mL) consisted of 3.5 mL of phosphate buffer (0.1 M pH 6.5), 0.2 mL enzyme 0.1 mL *O*-dianisidine (1mg / mL methanol) and the reaction was initiated by adding 0.2 mL of H_2O_2 (0.2 M) in a cuvette placed in spectrophotometer. The absorbance was measured at intervals of 10 seconds up to 3 minutes. The reagent blank contained all reagents except the substrate (H_2O_2). The enzyme activity was expressed as change in absorbance mg^{-1} protein m^{-1}.

3.3.2. Polyphenol oxidase (catachol oxidase) extraction and estimation

Extraction

Triplicate samples of about 0.5 g tissue (seed / leaf) were macerated in a pre-cooled mortar at 4ºC with cold sodium phosphate buffer (0.05 M, pH 6.0) at 1:5 ratio and with an equal quantity of acid purified sand. The homogenate was filtered through a double layer of cheesecloth. The filtrate was centrifuged in a Sorvall RC 5B refrigerated super speed centrifuge at 15000 x g for 10 min at 0 - 4ºC temperature. The supernatant was decanted to test tubes kept in ice bath and used as the enzyme source.

Assay

In *in vitro* enzyme assay, catechol was used as the substrate by adopting the procedure given by Simons and Ross (1971) with slight modifications. The initial rate of formation of quinone (product) was followed sprectrophotometrically by recording the absorbance at 495 nm using Shimadzu UV-160A automatic recording spectrophotometer.

Catechol (purified by sublimation) was dissolved in phosphate buffer (0.1 M, pH 6.0) to get a substrate concentration of 0.01 M. To 3mL of the substrate taken in a cuvette, 1 mL of enzyme was added, and increase in absorbance at 495 nm was recorded for 5 min at 10 seconds interval. The reagent blank constituted of the substrate solution without enzyme. The reaction rate was determined from the slope of the initial straight-line portion of the curve and was expressed as change in absorbance mg^{-1} protein m^{-1}.

3.3.3. Lipoxygenase extraction and estimation

Extraction

0.5 g of the tissue (seed / seedling / leaf) in triplicate were macerated in a pre-cooled mortar with an equal quantity of acid purified sand and 5 mL of cold sodium phosphate buffer (0.05 M, pH 6.5). The homogenate was filtered through a double layer of cheesecloth. The filtrate was centrifuged in a Sorvall RC 5B refrigerated super speed centrifuge at 15000 x g for 10 min at 0 - 4ºC temperature. The supernatant had a top layer of scum derived from fat extracted due to grinding the sample. Even though the scum was present in all the seed extracts; it did not interfere with other enzyme assays. However, in the lipoxygenase assay the lipid residues in the scum could interfere with activity determinations. In order to separate the clear supernatant from lipid layer an elegant method was developed. A glass tube with tapered and bent tip was inserted into the filtrate and sucked without damaging lipid layer. The supernatant was decanted to test tubes kept in ice bath and used as the enzyme source.

Preparation of substrate

10 mM sodium linoleate substrate was prepared as per the method described by Axelrod *et al*., (1981). 70 mg of linoleic acid was weighed into a stoppered-graduated test tube. An equal quantity (by weight) of Tween 20 and 4 mL of oxygen free distilled water were added. The mixture was homogenized by drawing it back and forth in a Pasture pipette, avoiding air bubble. Sufficient quantity (0.55 mL) of 0.5 N NaOH was added to yield a clear solution. The volume was made up to 25 mL using O_2-free distilled water. This mixture was divided into 2 mL portions in small screw cap vials flushed with N_2 and

then closed and sealed with paraffin wax strips. These vials were kept frozen until needed.

Assay

The enzyme activity was measured spectrophotometrically by measuring the formation of the conjugated diene hydroperoxide at 234 nm, by following the procedure described by Borthakur, Bhat and Ramadoss (1987).

The reaction mixture contained 2.7 mL of 0.2 M sodium phosphate buffer (pH 6.0) and 0.3 mL of 10 mM sodium linoleate. The reaction was initiated by adding 0.15 mL of enzyme extract and change in absorbance at 234 nm was recorded for 3 min at 10 seconds interval using Shimadzu UV-160A automatic recording spectrophotometer. The reagent blank constituted of the substrate solution without enzyme. The reaction rate was determined from the slope of the linear path of the curve and was expressed as change in absorbance mg^{-1} protein m^{-1}.

3.3.4. Phenylalanine ammonia-lyase extraction and estimation:

Extraction

The enzyme was extracted by grinding 1 g tissue (seed / leaf) with an equal quantity of acid purified sand and 5 mL cold sodium borate buffer (0.1 M, pH 8.8) containing 3mM β-mercaptoethanol, using a pre-cooled mortar and pestle. The homogenate was filtered through a double layer of cheesecloth and the filtrate was centrifuged at 20000 x g for 30 min at 0 - 4ºC temperature in a Sorvall RC 5B refrigerated super speed centrifuge. The supernatant was decanted to glass test tubes kept in ice bath and used as the enzyme source.

Assay

The enzyme activity was determined by measuring the quantity of product trans-cinnamic acid formed from *L*-phenylalanine sprectophotometrically at 290 nm, following the method described by Legrand *et al*., (1976).

The reaction mixture consisted of 2.3 ml sodium borate buffer (0.1 M, pH 8.8) and 0.1mL of same buffer containing 10 mM *L*-phenylalanine and 0.5 mL of enzyme extract. The reaction mixture was incubated for 2h in water bath maintained at 38ºC. The reaction was stopped by adding 0.1 mL of 10N H_2SO_4. The reagent blank was devoid of the substrate, keeping total volume of reaction mixture constant. The enzyme activity was expressed as µmoles of t-cinnamic acid formed mg^{-1} protein h^{-1}.

3.3.5. Ribonuclease extraction and estimation

Extraction

Around 0.5 g of the tissue (seed / leaf) in triplicate were frozen at –20ºC and then macerated in 5 mL of cold 50 mM ammonium acetate buffer (pH 5.4) and with an equal quantity of acid purified sand using a pre-cooled mortar and pestle. The homogenate was filtered through a double layer of cheesecloth. The filtrate was centrifuged in a Sorvall RC 5B refrigerated super speed centrifuge at 5000 x g for 10 min at 0 - 4ºC temperature. The supernatant was dilutedto get the resultant solution concentration of 20 mM ammonium acetate and used as the enzyme source. The pH was readjusted to 5.4.

Substrate purification

Yeast ribonucleic acid was purified by alcohol precipitation. 10 g of yeast ribonucleic acid was dissolved in 100 mL of distilled water and pH of the solution was

adjusted to 6.7 with 1N NaOH. Undissolved material was removed by centrifugation at 2000 rpm for 40 min. The clear supernatant was brought to pH 5.5 with acetic acid and to this, three volumes of absolute alcohol were added. After standing overnight at 4ºC, this was centrifuged at 5000 rpm for 10 min. The precipitate was washed successively with 70%, 95% and absolute alcohol followed by ether and then air-dried. This preparation was used as a substrate in the enzyme assay.

Assay

The activity of the enzyme was determined sprectophotometrically by reading the absorbance (at 260 nm) of the breakdown products, which were not precipitated by acid, basically following the procedure of Frisch-Niggermeyer and Reddi (1957) with slight modification.

A mixture of 0.1 mL of RNA solution (1 mg/mL) in 100 mM ammonium acetate buffer (pH 5.5), 0.1 mL of 100 mM ammonium acetate buffer (pH 5.5), 0.2 mL of distilled water and 0.1 mL of enzyme was incubated in water bath maintained at 37ºC for one hour. At the end of the reaction time, the tubes were transferred to an ice bath and 0.1 mL of cold 1M HCl was added to each tube to stop the reaction and to precipitate unhydrolysed material. After cooling for one hour in ice bath, the mixture was diluted with 1.9 mL of 200 mM HCl and centrifuged at 2000 rpm for 40 min. The supernatant was decanted to 1 cm quartz cells and absorbance was read at 260 nm. To the reagent blank HCl was added in the beginning and common reaction procedure was followed. The enzyme unit was defined in terms of increase in absorbance of reaction mixture over blank g^{-1} fresh weight h^{-1}.

3.3.6. Salicylic acid extraction, purification and estimation

Extraction

The method of salicylic acid extraction from plant tissue followed by Raskin *et al*., (1989) was adopted in the present study.

One to two grams of tissue (leaf/root) was ground with 90% methanol at 1:25 ratio with the help of mortar and pestle. The homogenate was centrifuged at 12000 x g for 15 min in Sorvall RC 5B refrigerated super speed centrifuge. The supernatant was decanted into a test tube. The pellet was resuspended in 100% methanol and the homogenate was re-centrifuged at 12000 x g for 15 min. The two supernatants were combined and dried by flushing N_2 gas at 40ºC. The residue was resuspended in 5 ml of 1:1 (vol/vol) mixture of ethyl acetate and cyclopentane containing 1% (vol/vol) isopropanol. The top organic phase was separated and was concentrated by flushing N_2 gas.

Separation of salicylic acid by thin layer chromatography

The thin layer chromatography method of salicylic acid separation as described by Rasmussen *et al*., (1991) was followed.

The concentrated extract was spotted into silica gel chromatography plates of 500 /u thickness prepared using CAMAG TLC plater. The chromatograms were developed in a mixture of solvents toluene: dioxane: acetic acid at 90: 25: 5 ratio (vol/vol). The run time varied from 1 ½ to 2 hours. Salicylic acid was visualized on the plate by viewing under UV light (352 nm). The fluorescent band corresponding to salicylic acid standard was eluted from the silica gel with 1 ml of methanol and centrifuged for 5 min at 1000

rpm. The supernatant was decanted into a graduated and stoppered test tube. The pellet was resuspended in 1 mL of methanol twice by repeating the process. The three eluates were combined and made up to final volume 3 mL with methanol and used for salicylic acid estimation.

Quantification of salicylic acid

The salicylic acid in methanol extract was quantified using spectrofluorophotometer (Shimadsu RF 5000), the procedure described by Rasmussen *et al*., (1991) with slight modification. Relative intensity was measured at excitation wavelength of λ 300 and extinction wavelength of λ 400 against methanol blank. Concentration of salicylic acid was calculated using the standard graph prepared with authentic salicylic acid standard. Internal standard was used every time for verification of results. The quantity of salicylic acid was indicated as μg g^{-1} fresh weight.

3.3.7. Study of protein band profile in seed

Defatting of seed

Defatting of seeds was done with hexane using "Refatech II 1050" extractor (Tecator Soxhlet apparatus) following the standard method of the American Oil Chemists (Anon., 1981). The ground samples of seeds (0.5 grams) of different varieties were weighed into Whatmann no. 2 filter paper packets. These packets containing seed powders were transferred to filter paper funnel and inserted into a thimble. A piece of absorbent cotton was placed on top of the thimble to distribute the solvent as it drips on the sample. The thimble was placed into an extraction tube fitted in the Soxhlet apparatus. Oil extraction was done for 2 hours taking 50 mL of hexane as solvent in the

extraction flask and heated using the inbuilt hot plate available in the apparatus. After oil extraction, the defatted powder was transferred to a Whatmann filter paper, allowed the solvent to evaporate and then kept in dessicator.

Protein separation

Polyacrylamide-sodium dodecyl sulphate slab gel electrophoresis of seed proteins was carried out adopting the procedure followed by Chakraborty (1988) with minor modifications. The defatted seed (0.2g) was extracted with 2mL of 0.05M sodium phosphate citrate buffer (pH 2.8) and centrifuged at 10000 x g for 30min. The supernatant was used as seed protein in electrophoresis. Electrophoresis was done using vertical slab containing 12% separation gel and 4% stacking gel. The protein extract was mixed at equal proportion with Tris HCl buffer (pH 8.8) containing glycerol and bromophenol blue as tracking dye. 75 µL of the sample buffer mixture was loaded per well and electrophoresed in a commercial apparatus for two hours using a current (100 V) of 12.14mA at start and 5.9 mA at the end.

Staining of protein bands

The separated protein bands were stained using two methods.

Coomassie blue staining of protein bands

The protein bands were visualized by coomassie brilliant blue staining in SDS-PAGE. Staining solution was prepared by dissolving 2 grams of coomassie brilliant blue R 250 in 100 mL of 45% methanol containing 10% acetic acid. The gels were soaked for several hours in the staining solution. Thereafter the gels were destained in 10% methanol containing 7% acetic acid.

Silver staining of protein bands

Gels were stained following high sensitivity silver staining method described by Gooderham (1984). The gel was placed in a glass dish and washed for more than 2 hours in "Solution A" containing 50% (v/v) methanol and 10% (v/v) acetic acid. The gel was further washed for more than 2 hours in "Solution B" containing 20% (v/v) methanol and 7% (v/v) acetic acid. The solution was then replaced with "Solution C" containing 10% (v/v) glutaraldehyde and the gel was washed for a further 30 min. Thereafter, the gel was washed overnight with at least four changes of distilled water. After washing, the ammonical silver nitrate solution (Solution D) was poured into the dish and the gel was vigorously agitated (~200 rpm) for 15 min. The gel was then briefly rinsed with two changes of distilled water and transferred to a clean gel dish between first and second rinses. The proteins were then visualized by the addition of "Solution E" containing 0.005% (w/v) citric acid, 0.019% (v/v) formaldehyde and gently rocking the gel dish. When the image has developed to the required intensity (within 5 min.) the gel was again washed in "Solution B" for about 10 min. before being finally stored in "Solution B" in dark at 4^{o}C. Leaving the gel for more than 2 hours in "Solution A" reversed the diffusion of bands due to increase in size of the gel during staining.

3.3.8. Protein estimation

Protein estimation in tissue extracts was done following the method of Bradford (1976) using bovine serum albumin as standard and coomassie brilliant blue G-250 reagent.

Preparation of reagent

Coomassie brilliant blue G-250 (25mg) was dissolved in 12.5 mL of 95% ethanol. To this solution, 25 mL of 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 250 mL using distilled water. The final concentrations of the reagent were 0.01% (w/v) coomassie brilliant blue G-250, 4.7% (w/v) ethanol and 8.5% (w/v) phosphoric acid.

Assay

To 0.1 mL of the enzyme extract 3 mL of the reagent was added and the contents were mixed by inversion. After 2 min, the absorbance was measured in 3 mL cuvettes against the reagent blank prepared from 0.1 mL of the appropriate buffer and 3 mL of protein reagent. The concentration of protein in the extract was estimated from the calibration curve prepared using bovine serum albumin as standard.

3.3.9. Tobacco mosaic virus isolation and inoculation

Source and maintenance

The common strain of tobacco mosaic virus (TMV) authenticated by tests, was isolated from the infected plants collected from CTRI Research Station, Hunsur and they were inoculated and maintained in *Nicotiana tabacum* cv. FCV Special which is susceptible to TMV (systemically infected).

Isolation and precipitation of virus

Precipitation of tobacco mosaic virus was carried out using polyethylene glycol 6000 (PEG) as described by Hebert (1963). Leaves of systemically infected *Nicotiana tabacum* cv. FCV Special were macerated with equal weight of water in a mortar. The

homogenate was centrifuged at 10000 rpm for 10 min. The supernatant was heated at 60ºC for 10 min and again centrifuged at 1000 rpm for 10 min. The supernatant was mixed with an equal volume of 0.2M NaCl solution containing 8% PEG to make the final concentration of 0.1M NaCl & 4% PEG. After 10 minutes the mixture was centrifuged at 10000 rpm for 10 min. The pellet was resuspended in water and assayed for infectivity by inoculating to the leaves of *Nicotiana glutinosa*, which is resistant to TMV showing necrotic local lesions on infection.

Inoculation of virus

Nearly fully expanded upper most non-senescing leaves were abraded with wet carborundum (200 to 400 mesh) and inoculated with 200 µL (containing approximately 20 to 30 µg/mL) of a sterile aqueous suspension of purified TMV. Mock-inoculated leaves were abraded and inoculated with water alone by gently rubbing.

3.4. Experimental Design &Statistical Analysis

The experiment was conducted according to factorial based completely randomized design with 3 replications. The results in tables are presented as arithmetical means ± SE of 3 measurements of 3 replications. Data were analysed by 2 or 3 factor (as the case may be) ANOVA and significant treatment effects were determined by LSD at P = 0.05.

In the breeding experiment, chi-square tests were used to determine the goodness to fit of the observed number of plants to the predicted segregation ratios of F_2& back cross with susceptible and resistant parent (BC-1 & BC-2) progenies to establish the mode of inheritance. The chi-square value was calculated using standard procedure.

4. Results

4. RESULTS

4.1. Peroxidase

Peroxidase activity during seed germination

There was no detectable peroxidase (POX) activity in seeds of the three varieties. The enzyme was activated only from 4th day of sprouting in all the three varieties tested (Table 4.1.1)and thereafter increase in the activity was noticed. Comparison of the enzyme levels revealed that the resistant variety contained more enzyme activity as compared to tolerant and susceptible varieties. This higher level was always seen in all the days of germination tested. It is evident from Table 4.1.2 there is a sudden spurt in the activity between 4th to 6th day of germination. The enzyme profile thereafter showed a zigzag pattern of induction as revealed from Fig. 4.1.1. This pattern is expected in peroxidase activity as reported from the literature.

Table 4.1.1. Peroxidase activity at different stages of seed germination in *Nicotiana tabacum* cultivars

Variety / Days	Peroxidase activity **		
	FCV Special (S)	FCH 6248 (T)	Va.770 (R)
2	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
4	0.5418 ± 0.0570	0.5732 ± 0.0060	1.1455 ± 0.0780
6	1.5202 ± 0.0540	1.5650 ± 0.0160	2.5042 ± 0.0460
8	1.7924 ± 0.0780	1.7759 ± 0.0520	4.0731 ± 0.1130
10	6.1316 ± 0.1960	4.7955 ± 0.3910	6.1616 ± 0.4860
12	6.7757 ± 0.2770	6.7169 ± 0.0890	9.6474 ± 0.1010
14	6.4624 ± 0.0770	8.8832 ± 0.3140	11.5548 ± 0.0920
CD at P= 0.05	0.2746		

(R) – Resistant; (T) – Tolerant; (S) – Susceptible

**Change in absorbance at 430 nm mg^{-1} protein min^{-1}

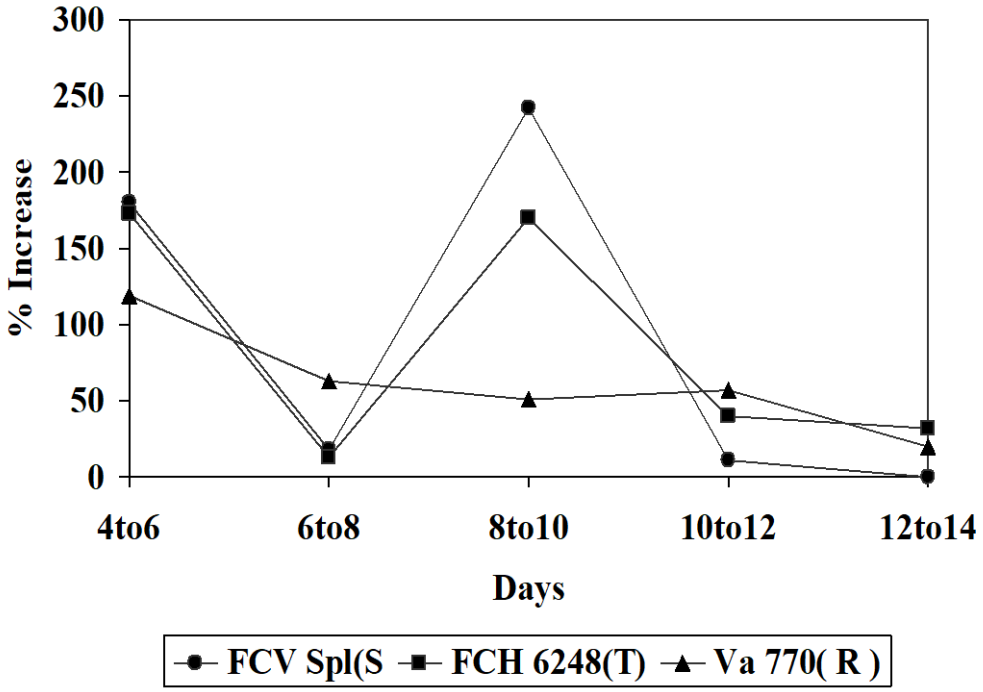


Fig. 4.1.1. Kinetics of peroxidase activity induction during seed germination

Table 4.1.2. Induction of peroxidase enzyme during seed germination

Variety	Difference in activity (%)				
	Day 4 to 6	Day 6 to 8	Day 8 to 10	Day 10 to 12	Day 12 to 14
FCV Special (S)	+180	+18	+242	+11	-5
FCH 6248 (T)	+173	+13	+170	+40	+32
Va.770 (R)	+119	+63	+51	+57	+20

(R) – Resistant; (T) – Tolerant; (S) - Susceptible

Effect of tobacco mosaic virus infection on peroxidase activity

The enzyme profile was also checked after TMV inoculation in all the varieties using 50-day-old seedlings. The Table 4.1.3 indicates that there is a higher induction in both the two resistant varieties viz. Va 770 and *Nicotiana glutinosa* as compared to tolerant and susceptible varieties. However, the data presented in graph (Fig. 4.1.2, 4.1.3 & 4.1.4) again shows the zigzag pattern of enzyme induction. It may be concluded that

even though, TMV infection enhances peroxidase activity, it follows the zigzag trend, which was noticed even in the absence of TMV infection as shown in Fig. 4.1.4, and hence it is growth related.

Table 4.1.3. Peroxidase activity induction on viral interaction in *Nicotiana tabacum* cultivars

Variety	Peroxidase activity **		
	Un inoculated control	Mock inoculated control	TMV inoculated
FCV Special (S)	23.27 ± 1.30	24.93 ± 1.51	24.81 ± 0.77
FCH 6248 (T)	23.51 ± 0.87	24.40 ± 0.70	24.31 ± 1.49
Va.770 (R)	22.93 ± 0.74	23.29 ± 0.67	27.07 ± 0.35
N. glutinosa	30.37 ± 0.93	34.54 ± 0.71	40.27 ± 1.01
CD at P= 0.05	1.42		

(R) – Resistant; (T) – Tolerant; (S) - Susceptible

** Change in absorbance at 430 nm mg^{-1} protein min^{-1}

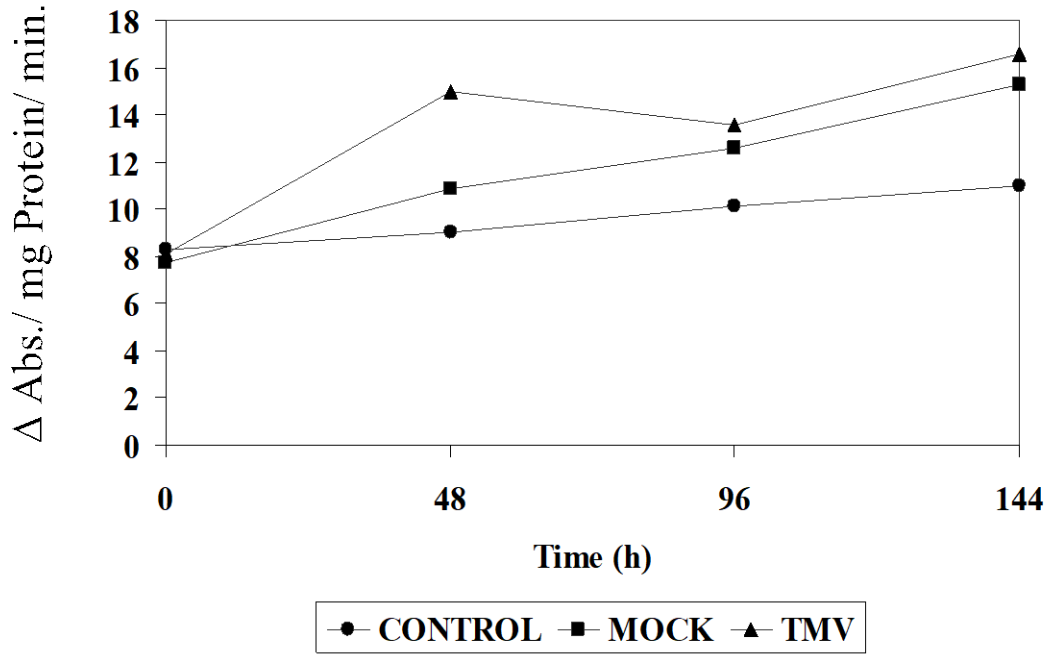


Fig. 4.1.2. Kinetics of peroxidase activity during TMV infection in FCV Special

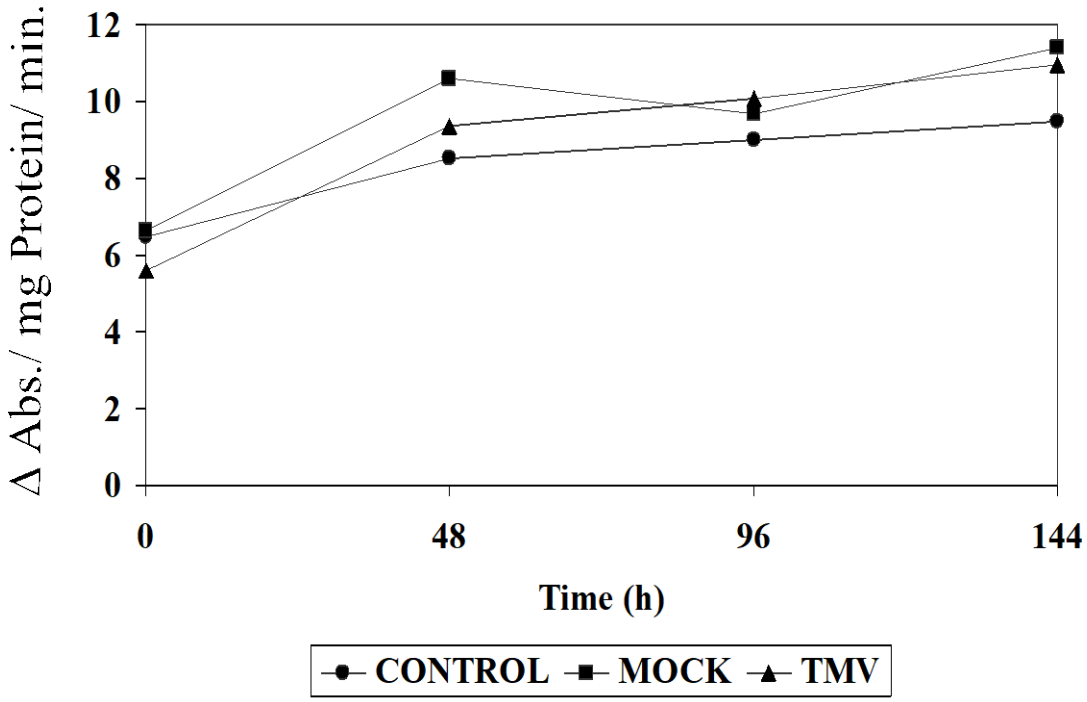


Fig. 4.1.3. Kinetics of peroxidase activity during TMV infection in FCH 6248

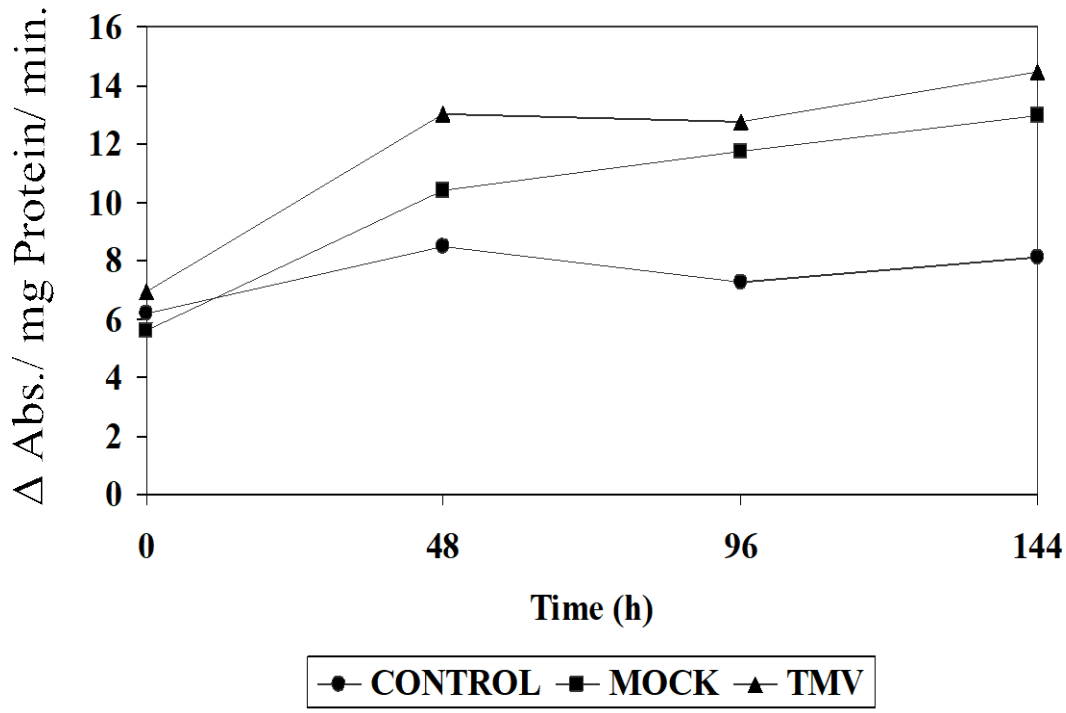


Fig. 4.1.4. Kinetics of peroxidase activity during TMV infection in Va 770

Hence, depending only on POX induction as a biochemical parameter for resistance may not be explicit. The enzyme induction depends on the total stress among which TMV infection may be one. Therefore, the induction reveals the cumulative impact of the stress rather than exclusively due to infection. To further confirm the influence of other physiological stress on enzyme induction, the enzyme levels in leaves of varied age were measured.

Peroxidase activity in leaves

In all the 3 varieties tested, leaves at the bottom of the plant had higher activity compared to the middle and leaves on top of the plant. The activity level in bottom leaf was 11, 24 &32-fold higher than that of top leaves in susceptible, resistant and tolerant varieties respectively. In this case, the higher activity seen in the bottom leaves is due to stress by senescence. Therefore, the newly formed leaves on top have less stress due to senescence hence the least activity. The data presented in Table 4.1.4 supports the view described above.

Table 4.1.4. Peroxidase activity at different leaf positions in *Nicotiana tabacum* cultivars

Leaf Position / Variety	Peroxidase activity **		
	Bottom	**Middle**	**Top**
FCV Special (S)	9.1618 ± 1.44	3.1700 ± 0.04	0.8431 ± 0.21
FCH 6248 (T)	33.6369 ± 5.53	4.4001 ± 0.76	1.4018 ± 0.54
Va.770 (R)	63.3097 ± 9.28	4.9386 ± 0.57	1.9812 ± 0.19
CD at P= 0.05	6.2682		

(R) – Resistant; (T) – Tolerant; (S) - Susceptible
** Change in absorbance at 430 nm mg^{-1} protein min^{-1}

4.2. Polyphenol Oxidase

Polyphenol oxidase activity during seed germination

Polyphenol oxidase (PPO) activity was tested in the seeds of susceptible, tolerant and resistant varieties. There was no significant activity found in any variety. The pattern of enzyme activity during germination of seed is shown in table 4.2.1. In all the three varieties, the PPO activity appeared on day 4 and increased thereafter to attain peak on day 12. On the 14th day the enzyme activity was reduced. Comparison of the maximum activity between the varieties showed highest of 0.4 units in the susceptible variety, while, it was only 0.37 units in resistant and the least (0.17 units) in tolerant variety. PPO has multiple roles in plants. One of the functions is to meet the energy demand. Since the susceptible variety has faster growth requiring higher energy demands, higher activity was observed in this variety.

Table 4.2.1. Polyphenol oxidase activity at different stages of seed germination in *Nicotiana tabacum* cultivars

Variety / Days	Polyphenol oxidase activity **		
	FCV Special (S)	FCH 6248 (T)	Va.770 (R)
2	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
4	0.0256 ± 0.0015	0.0262 ± 0.0030	0.0475 ± 0.0024
6	0.0727 ± 0.0045	0.0332 ± 0.0006	0.0884 ± 0.0076
8	0.0995 ± 0.0075	0.0391 ± 0.0043	0.1022 ± 0.0101
10	0.2451 ± 0.0328	0.1162 ± 0.0339	0.1642 ± 0.0408
12	0.4086 ± 0.0430	0.1691 ± 0.0088	0.3652 ± 0.0091
14	0.3299 ± 0.0106	0.1403 ± 0.0038	0.2696 ± 0.0006
CD at P= 0.05	0.0285		

(R) – Resistant; (T) – Tolerant; (S) - Susceptible
**Change in absorbance at 495 nm mg^{-1} protein min^{-1}

Polyphenol oxidase activity in leaves

Perusal of the data given in Table 4.2.2 supports the view stated above. In this study, PPO activities in bottom, middle and top leaves of all the varieties were compared. The energy demand in the older bottom leaves is less as compared to the growing leaves at the top. Accordingly, highest level of PPO was found in the younger top leaves followed by the least activity in the oldest bottom leaves, while the activity in middle leaves fell in between.

Table 4.2.2. Polyphenol oxidase activity at different leaf positions in *Nicotiana tabacum* cultivars

Leaf Position / Variety	Polyphenol oxidase activity **		
	Bottom	**Middle**	**Top**
FCV Special (S)	0.0252 ± 0.0081	0.0302 ± 0.0084	0.0549 ± 0.0046
FCH 6248 (T)	0.0286 ± 0.0025	0.0453 ± 0.0098	0.0521 ± 0.0114
Va.770 (R)	0.0438 ± 0.0080	0.0560 ± 0.0083	0.0703 ± 0.0054
CD at P= 0.05	0.0134		

(R) – Resistant; (T) – Tolerant; (S) - Susceptible

** Change in absorbance at 495 nm mg^{-1} protein min^{-1}

Effect of tobacco mosaic virus infection on polyphenol oxidase activity

The role of polyphenol oxidase in protecting the plants against pathogen infection and/or wound healing was tested. It is interesting to note (Table 4.2.3) highest induction was found due to TMV infection in the susceptible variety. Whereas, the resistant variety (Va 770) showed less activity and the least enzyme level was recorded in the highly resistant *Nicotiana glutinosa*. By definition, a susceptible variety has the least defense mechanism against the invasion of pathogen and in order to defend infection; it should

induce the maximum quantity of enzyme. This explains the highest level observed in susceptible plant. On the same note it can be argued, the highly resistant genotype has already the built-in mechanism to prevent infection and therefore, it needs to induce least quantity of PPO. That is exactly found in the highly resistant *N. glutinosa.*

Table 4.2.3. Polyphenol oxidase activity on viral interaction in *Nicotiana tabacum* cultivars

Variety	Polyphenol oxidase activity **		
	Un inoculated control	Mock inoculated control	TMV inoculated
FCV Special (S)	0.1246 ± 0.0042	0.1848 ± 0.0047	0.4265 ± 0.0119
FCH 6248 (T)	0.0842 ± 0.0056	0.1144 ± 0.0094	0.1506 ± 0.0102
Va.770 (R)	0.0667 ± 0.0053	0.1217 ± 0.0036	0.1774 ± 0.0036
N. glutinosa	0.0151 ± 0.0042	0.0228 ± 0.0060	0.0220 ± 0.0028
CD at P= 0.05	0.0076		

(R) – Resistant; (T) – Tolerant; (S) - Susceptible

** Change in absorbance at 495 nm mg^{-1} protein min^{-1}

Comparison of the results, bring out the fact that polyphenol oxidase is an enzyme having multiple role in the welfare of plant. It is necessary to promote growth, in the supply of anabolic metabolites in younger leaves, as exemplified by the high metabolic turn over. This enzyme is also involved in protecting the plants against invasion by pathogen such as TMV. Yet another role of PPO in wound healing is also revealed in the present experiment. This is evident by the increase in enzyme activity due to pricking in mock-inoculated plants. Perusal of the response due to pricking as compared to control leaves clearly shows higher activity. This elevated level is probably involved in healing the wound formed as a result of pricking.

In conclusion, the study reveals that although polyphenol oxidase plays a role in disease resistance against TMV, it is not exclusive to infection. Therefore, relaying only on the enzyme PPO as a biochemical tool to disease resistance may not be appropriate.

4.3. Lipoxygenase

Lipoxygenase activity in seed

Lipoxygenase (LOX) was assayed in seeds of TMV susceptible (FCV Special), tolerant (FCH 6248) and resistant (Va 770) varieties (Table 4.3.1). The activity was 0.46 units (Δ absorbance $_{234\ nm}$ / mg protein / min) in susceptible variety and lowest among the three. In tolerant and resistant varieties, the activity measured in control samples were 67% &107% higher over that of susceptible and also made in water soaked seeds and also made in powdered & defatted seed because, the fat content used to interfere with enzyme extraction and activity determination. There was no appreciable increase in activity by imposing treatments. However, the difference among varieties was maintained in both the treatments.

Table 4.3.1. Lipoxygenase activity in seeds of different *Nicotiana tabacum* cultivars

Variety	Lipoxygenase activity **		
	Control	Soaked	Defatted
FCV Special (S)	0.4615 ± 0.06	0.4709 ± 0.10	0.5130 ± 0.04
FCH 6248 (T)	0.7705 ± 0.05	0.7970 ± 0.01	0.8195 ± 0.06
Va.770 (R)	0.9575 ± 0.04	0.9562 ± 0.04	1.0057 ± 0.05
CD at P= 0.05	0.0940		

(R) – Resistant; (T) – Tolerant; (S) - Susceptible

**Change in absorbance at 234 nm mg^{-1} protein min^{-1}

To validate the hypothesis higher enzyme activity in TMV resistant variety than that in susceptible, levels in 13 TMV resistant genotypes were measured (Fig. 4.3.1). Activity varied from 0.86 units to 2.07 units among the genotypes assayed. However, the levels of LOX activity in all the 13 genotypes were higher than that recorded in susceptible varieties FCV Special, Bhavya and Swarna. The activity in tolerant variety (FCH 6248) was below the level of variety Va 770.

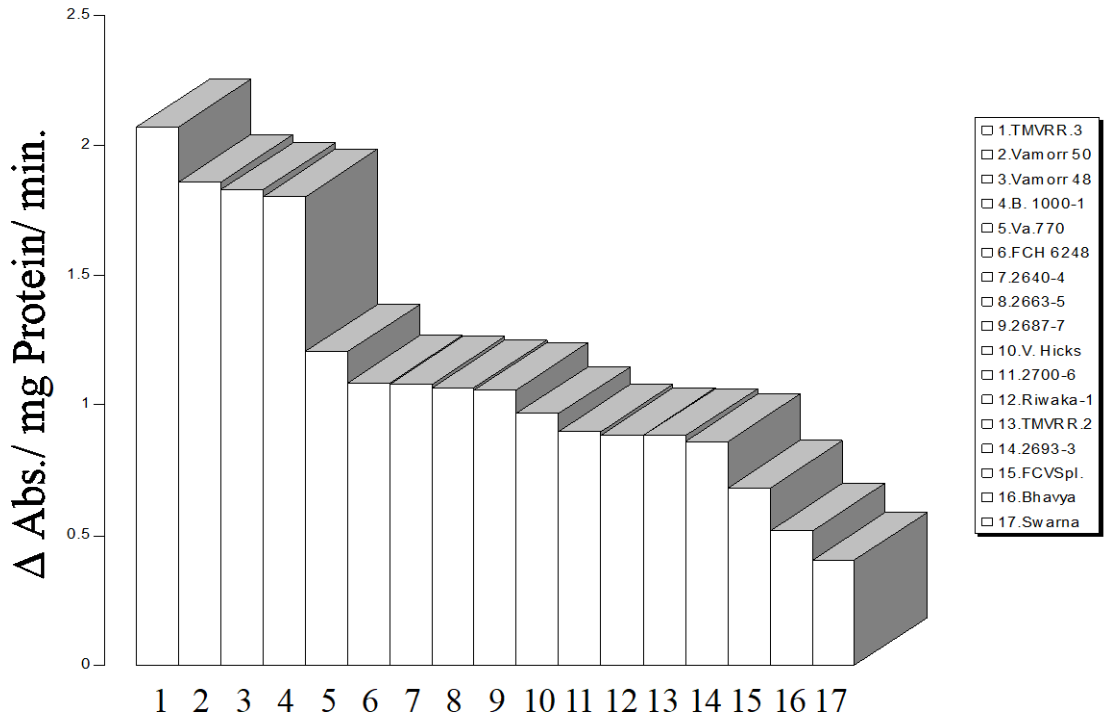


Fig. 4.3.1. Lipoxygenase level in seeds of different genotypes

Lipoxygenase activity during seed germination

Lipoxygenase activity was induced during seed germination in all the three varieties. Increase in enzyme activity was recorded up to 12 days, and declined thereafter (Fig. 4.3.2). On day 12 highest activity of 5.5 units was recorded in susceptible variety followed by tolerant variety (5.3) and lowest level of 3.9 units in resistant variety.

However, the kinetics of induction revealed a different pattern. On day 2 maximum enzyme activity was noticed in the resistant variety and the minimum was recorded in the susceptible variety. The increase in the enzyme activity from day 2 to day 12 in susceptible variety therefore is 9.6 folds, whereas in resistant variety it was only 4.4 folds. Higher induction in susceptible variety probably is related to the faster seedling growth of this variety, due to mobilization of stored nutrients from the seed to the developing seedling. The fall in the activity after 12 days supports this hypothesis because; the seedling growth beyond 12 days does not depend on the nutrients from the seed. It is interesting that the rate of fall of activity in the resistant variety after 12 days is less and the decline is only 35% (Table 4.3.2) as compared to the 58% decrease in the susceptible variety.

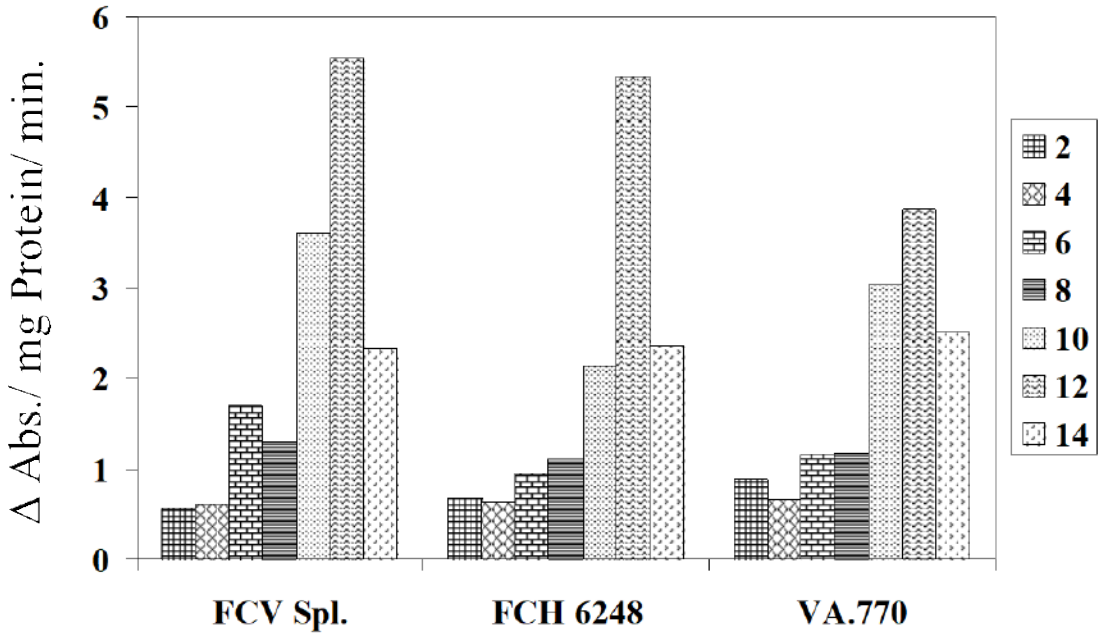


Fig. 4.3.2. Lipoxygenase level during seed germination in *N. tabacum* cultivars

Table 4.3.2. Lipoxygenase activity levels at different stages of seed germination in *Nicotiana tabacum* cultivars

Variety / Days	Lipoxygenase activity **		
	FCV Special (S)	FCH 6248 (T)	Va.770 (R)
2	0.5552 ± 0.001	0.6660 ± 0.011	0.8869 ± 0.015
4	0.6076 ± 0.029	0.6220 ± 0.017	0.6621 ± 0.080
6	1.7079 ± 0.017	0.9482 ± 0.037	1.1517 ± 0.049
8	1.2994 ± 0.019	1.1097 ± 0.109	1.1653 ± 0.042
10	3.6111 ± 0.187	2.1387 ± 0.055	3.0359 ± 0.209
12	5.5336 ± 0.064	5.3419 ± 0.154	3.8731 ± 0.307
14	2.3268 ± 0.086	2.3579 ± 0.089	2.5126 ± 0.285
CD at P= 0.05	0.2032		

(R) – Resistant; (T) – Tolerant; (S) - Susceptible
**Change in absorbance at 234 nm mg^{-1} protein min^{-1}

The kinetic data comparison after 12 days implies that the LOX in resistant variety has some other function rather than the mere growth related activity. Additional function may be perhaps related to resistance to TMV infection (involved in resistance mechanism).

Effect of tobacco mosaic virus infection on lipoxygenase activity

LOX activity in response to virus inoculation was studied using 20-day-old seedlings of susceptible (FCV Special), tolerant (FCH 6248) and resistant (Va 770) (Table 4.3.3). The kinetics of induction in uninoculated control did not show significant change in the enzyme activities between the varieties studied. The activity was about 2 units in all the varieties at all times tested. But the kinetic pattern changed due to TMV

infection. In the resistant variety, the activity doubled in 24 hours after infection. While, the increase was not so significant in tolerant and susceptible varieties.

Table 4.3.3. Changes in lipoxygenase activity on TMV inoculation in *Nicotiana tabacum* cultivars

Varieties / Time (h)	Lipoxygenase activity (Δ Abs $_{234\ nm}$ / mg protein / min)		
	Un inoculated control	Mock inoculated control	TMV inoculated
Va 770 (R):			
0	2.5463 ± 0.25	2.4378 ± 0.39	2.3698 ± 0.24
24	2.6335 ± 0.07	3.6724 ± 0.11	4.9200 ± 0.17
48	2.0184 ± 0.19	3.4143 ± 0.34	4.8797 ± 0.15
CD at P= 0.05	0.3637		
FCH 6248 (T):			
0	2.1053 ± 0.21	2.2106 ± 0.14	2.1059 ± 0.14
24	2.0079 ± 0.12	2.9873 ± 0.09	3.0119 ± 0.27
48	2.2304 ± 0.21	2.1872 ± 0.16	2.9331 ± 0.14
CD at P= 0.05	0.2914		
FCV Special (S):			
0	1.9645 ± 0.12	2.0145 ± 0.10	2.1126 ± 0.25
24	2.0163 ± 0.10	2.6026 ± 0.17	2.5804 ± 0.09
48	1.8419 ± 0.08	1.9581 ± 0.16	2.7796 ± 0.45
CD at P= 0.05	0.3691		

(R) Resistant; (T) Tolerant; (S) – Susceptible

In Fig.4.3.3 the actual increase in activity due to viral infection has been calculated by subtracting the stimulation due to pricking in the mock inoculation. There has been a 34% increase in the LOX levels over the positive control (mock inoculation) in resistant variety, while; it was negligible in the other two varieties tested. Here again, a

definite enhancement of LOX level in resistant variety is seen due to viral infection, thereby indicating its role in conferring resistance to TMV infection.

Effect of TMV inoculation on lipoxygenase was also studied using 50-day-old seedlings. LOX activity was checked 5 days after inoculation and the data presented in Table 4.3.4. There is a significant increase in activity in resistant variety as compared to susceptible variety and the increasing trend confirms the results obtained by using 20-day-old seedlings. It may be observed that the enzyme activity in resistant variety Va 770 was similar to *Nicotiana glutinosa* from which TMV resistance is derived through breeding. This shows that the enzyme behaviour in the progeny (Va 770) is inherited along with resistance from *N. glutinosa*. And therefore, it may be surmised that higher LOX activity is associated with resistance and is inherited.

Table 4.3.4. Lipoxygenase activity induction on viral interaction in *Nicotiana tabacum* cultivars

Variety	Lipoxygenase activity **		
	Un inoculated control	**Mock inoculated control**	**TMV inoculated**
FCV Special (S)	0.8813 ± 0.142	1.0012 ± 0.203	1.0003 ± 0.025
FCH 6248 (T)	0.8594 ± 0.096	0.9482 ± 0.075	1.0832 ± 0.129
Va.770 (R)	0.6937 ± 0.040	0.7213 ± 0.006	1.2714 ± 0.065
N. glutinosa	0.7282 ± 0.016	0.8096 ± 0.068	1.3683 ± 0.053
CD at P= 0.05	0.1588		

(R) – Resistant; (T) – Tolerant; (S) - Susceptible
** Change in absorbance at 234 nm mg^{-1} protein min^{-1}

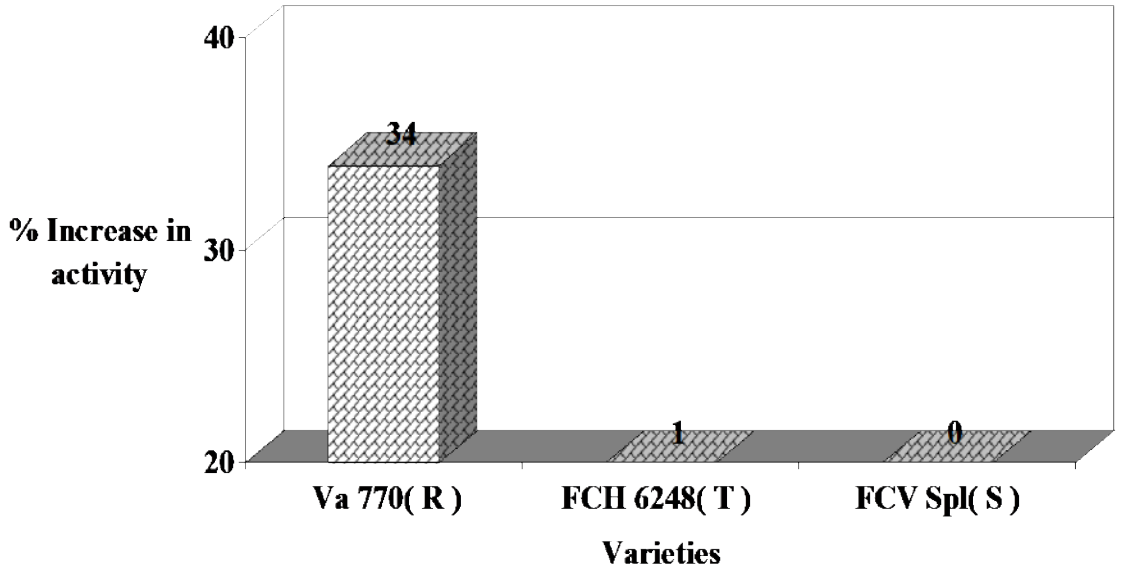


Fig. 4.3.3. Increase in lipoxygenase activity due to TMV infection in different varieties

4.4. Phenylalanine Ammonia-Lyase

Quantification of t–cinnamic acid

Enzyme activity of phenylalanine ammonia - lyase (PAL) was determined by measuring quantity of the product t–cinnamic acid formed from L-phenylalanine. For the spectrophotometric estimation of t – cinnamic acid a standard graph was prepared. The absorbance values obtained at 290 nm showed linearity in the range of 0.340 to 2.495 for the concentrations 0.1 to 0.8 µmoles in 3 mL assay mixture (Fig. 4.4.1). The constants for K and B were determined as 0.3136 and –0.0101 respectively. Using these K and B values and substituting the absorbance of unknown sample in equation "concentration (µmole) = K x Abs.290 nm + B" the concentration of t-cinnamic acid was calculated for experimental samples.

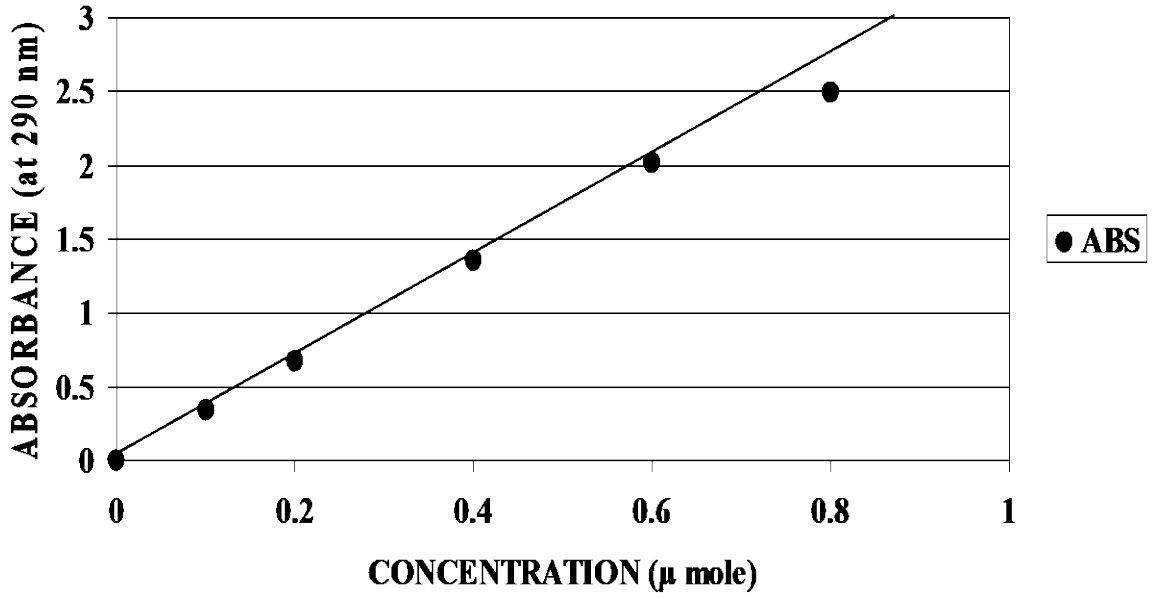


Fig. 4.4.1. Calibration curve for the estimation of t-cinnamic acid

Enzyme activity elicitation by TMV inoculation in resistant cultivar

In order to ascertain the activation of PAL on tobacco mosaic virus inoculation, levels of enzyme activity in fully expanded leaves of grown up resistant variety (Va 770), was measured at 12 hours after inoculation. A leaf of the same age from the same stalk position of different plant was mock inoculated with sterile water to serve as positive control and leaf from an uninoculated plant was taken as control check. The results shown in Table 4.4.1 clearly indicated significant differences in enzyme levels. Virus inoculation elicited 2.6-fold increase in the enzyme activity as compared to uninoculated leaves. While, mechanical injury (due to mock inoculation) induced only 1.3-fold increase in the enzyme activity. The higher level of PAL is perhaps indicative of its role in resistance to TMV disease in this variety. This hypothesis paved way for comparative studies on enzyme behaviour in susceptible, tolerant and resistant cultivars.

Table 4.4.1. Induction of phenylalanine ammonia-lyase activity by TMV inoculation in leaves of resistant variety

Treatment	PAL activity **
Un inoculated control	0.2598 ± 0.002
Mock inoculated control	0.3424 ± 0.010
TMV inoculated	0.6696 ± 0.120

**μmoles t- cinnamic acid mg^{-1} protein h^{-1}

Alteration in enzyme activity by TMV inoculation at seedling stage in susceptible, tolerant and resistant cultivars

The seedlings (20 day old) were subjected to the host – virus interaction studies. Comparison of data (Table 4.4.2) in controls indicates higher level of enzyme activity in tolerant variety as compared to that in resistant and susceptible varieties. While the initial level of enzyme product was 5 and 9 μmoles / mg protein / min in resistant and susceptible cultivars respectively, it was 1.5 to 2-fold higher (14.33 μmoles t.c.a / mg protein / min) in tolerant variety. However, the enzyme level did not change much in all three varieties studied during the period of experimentation (72 hours).

As evident from Fig. 4.4.2, with in a day after TMV inoculation, the PAL level in resistant variety increased 3-fold from the initial 5.97 units to 20.53 units. This tendency continued and at 72 hours post-inoculation the activity was 55.19, there by registering a 9-fold increase. In tolerant and susceptible varieties also there was an increase in PAL levels after viral infection. However, this increase was only 2-fold.

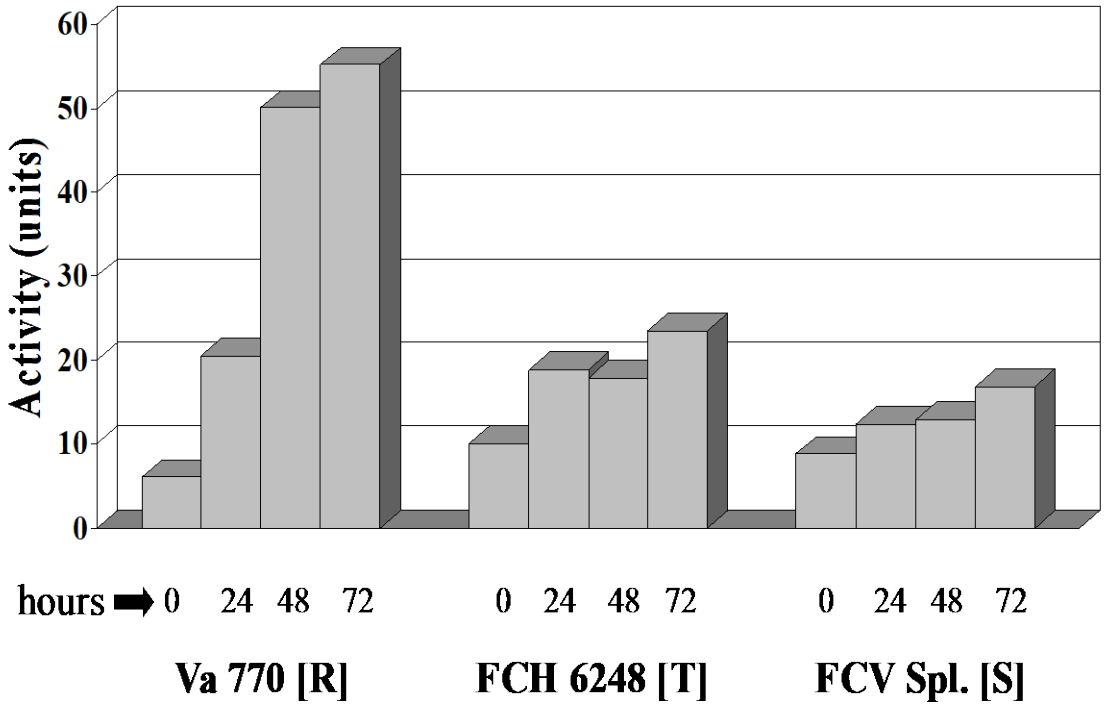


Fig. 4.4.2. Changes in phenylalanine ammonia- lyase activity on TMV inoculation in different varieties

Since PAL is known to be involved in altering plant defense mechanism, the significant increase in enzyme activity in the resistant variety due to viral infection confirms that the plant mounts its defense mechanism by the induction of PAL, which is a regulatory enzyme in phenyl propanoid pathway. Since the artificial inoculation involved mechanical injury of leaves, which may also induce defense mechanism of the plant to heal the injury. This again was verified by the mock inoculation experiments where the leaves were rubbed with carborundum. The results in Table 4.4.2 shows indeed increased activity of PAL due to mechanical injury.

Table 4.4.2. Time course of changes in phenylalanine ammonia-lyase activity on TMV inoculation in *Nicotiana tabacum* cultivars

Varieties/ Time (h)	PAL activity **		
	Un inoculated control	Mock inoculated control	TMV inoculated
Va 770 (R):			
0	05.08 ± 0.59	05.17 ± 0.49	05.97 ± 0.80
24	06.31 ± 0.61	10.29 ± 1.44	20.53 ± 1.46
48	05.21 ± 1.34	15.53 ± 0.75	50.02 ± 1.83
72	05.88 ± 2.18	23.10 ± 2.01	55.19 ± 0.57
CD at P= 0.05	2.21		
FCH 6248 (T):			
0	14.33 ± 1.05	12.08 ± 1.91	10.01 ± 0.84
24	13.02 ± 0.77	10.06 ± 1.52	18.83 ± 0.91
48	12.95 ± 1.86	18.93 ± 1.12	17.78 ± 1.49
72	12.07 ± 1.48	21.20 ± 2.56	23.49 ± 0.92
CD at P= 0.05	2.47		
FCV Special (S):			
0	08.89 ± 0.72	08.08 ± 0.65	08.78 ± 0.64
24	10.05 ± 0.28	08.90 ± 0.96	12.32 ± 1.31
48	11.34 ± 0.63	10.61 ± 2.21	12.89 ± 1.66
72	10.35 ± 0.98	11.48 ± 2.60	16.82 ± 1.20
CD at P= 0.05	2.24		

(R) – Resistant; (T) – Tolerant; (S) - Susceptible
**µmoles t- cinnamic acid mg^{-1} protein h^{-1}

However, the increase was only 2 to 3 folds. It is to be noted that this 2 to 3-fold enhancement is common to all the three varieties tested. The nine-fold increase seen in the resistant variety is therefore due to viral inoculation and this increase leads to resistance.

Experiments were repeated using 30-day-old seedlings in order to ascertain the behaviour of the enzyme in older plants. The results presented in Table 4.4.3 and figures reveal the same trend. TMV inoculation triggered the PAL activity. However, the level of

increase was lower (4-fold) as compared to 9-fold enhancement noticed in 20-day-old resistant plant. In general, perusal of the data suggests overall decrease in the PAL levels in all the three varieties tested. The studies reveal that even though the age of seedlings for testing the enzyme activity is not crucial, it is desirable to select younger seedlings where the changes are profound. It is also interesting to note that PAL activity is nil / insignificant in the seeds of all varieties.

Table 4.4.3. Kinetics of phenylalanine ammonia- lyase activity on TMV inoculation in *Nicotiana tabacum* cultivars

Varieties/ Time (h)	PAL activity **		
	Un inoculated Control	Mock inoculated Control	TMV inoculated
Va 770 (R):			
0	06.17 ± 1.38	06.29 ± 0.75	06.23 ± 1.06
24	06.60 ± 0.89	07.19 ± 0.74	10.30 ± 0.34
48	06.42 ± 1.31	10.64 ± 1.07	24.33 ± 0.73
72	05.96 ± 0.93	16.84 ± 0.93	27.96 ± 1.01
CD at P= 0.05	1.63		
FCH 6248 (T):			
0	06.90 ± 0.64	07.63 ± 2.23	06.91 ± 0.31
24	05.82 ± 0.62	11.12 ± 2.25	14.91 ± 1.15
48	06.48 ± 1.75	15.02 ± 2.02	14.96 ± 0.85
72	05.41 ± 1.29	14.30 ± 0.51	10.49 ± 0.94
CD at P= 0.05	2.33		
FCV Special (S):			
0	07.05 ± 0.44	08.34 ± 0.17	08.58 ± 1.22
24	09.11 ± 0.31	10.38 ± 1.07	10.04 ± 1.67
48	07.96 ± 0.75	12.66 ± 1.09	13.00 ± 0.79
72	06.24 ± 1.81	13.96 ± 0.97	13.72 ± 1.00
CD at P= 0.05	1.78		

(R) Resistant; (T) Tolerant; (S) Susceptible**/u moles t- cinnamic acid mg^{-1} protein h^{-1}

4.5. Ribonuclease

Enzyme activity in seed

Ribonuclease activity in seeds of susceptible, tolerant and resistant cultivars was measured. In seeds of both susceptible and tolerant varieties the enzyme activity was about 70 units / g of seeds. However, there was significantly higher activity (86 units per g of seeds) in resistant variety (Table 4.5.1).

Table 4. 5.1. Ribonuclease activity in seeds of different *Nicotianatabacum* cultivars

Variety	RNase activity **
FCV Special (S)	70.7 ± 3.3
FCH 6248 (T)	73.8 ± 2.3
Va.770 (R)	86.1 ± 1.1
CD at P= 0.05	4.8

(R) – Resistant; (T) – Tolerant; (S) - Susceptible

** Change in absorbance at 260 nm g^{-1} fresh weight h^{-1}

Change in ribonuclease enzyme activity due to viral infection in susceptible, tolerant and resistant cultivars

Ribonuclease activity of 20-day-old susceptible cultivar increased from the initial (soon after inoculation) 26 units (Change in absorbance at 260 nm g^{-1} fresh weight h^{-1}) to 46.67 units after 192 hours post inoculation, thereby registering about 80% increase (Table 4.5.2).

The results presented in Table 4.5.2 also indicate that in tolerant variety the initial activity was lower than that in the susceptible plant. Here again the activity increased from 16.6 units to the peak value of 23 units on day 4. The activity gradually decreased thereafter. In the resistant variety, highest activity of 42.6 units was measured on the 4th day, which was two folds increase over the initial level of 21 units.

Table 4.5.2. Induction of ribonuclease activity on TMV inoculation in *Nicotiana tabacum* cultivars

Time (h)	RNase activity **		
	FCV Special (S)	**FCH 6248 (T)**	**Va.770 (R)**
0	26.08 ± 3.89	16.62 ± 1.55	21.05 ± 6.34
48	35.30 ± 1.10	22.60 ± 0.81	34.87 ± 3.03
96	43.53 ± 0.70	23.35 ± 0.35	42.59 ± 4.58
144	44.67 ± 1.43	20.49 ± 3.92	41.82 ± 1.01
192	46.67 ± 2.15	20.62 ± 1.01	39.15 ± 7.64
CD at P= 0.05	5.31		

(R) – Resistant; (T) – Tolerant; (S) - Susceptible

** Change in absorbance at 260 nm g^{-1} fresh weight h^{-1}

Comparison of results on the varietal basis (Fig.4.5.1) shows that the susceptible variety had the highest activity and decreasing trend in other varieties following the pattern FCV Special > Va 770 > FCH 6248 throughout the study.

To understand the differential response of genotypes to viral infection, a comparative study of ribonuclease activity in uninoculated, mock inoculated and TMV inoculated plants was conducted using 50 days old seedlings.

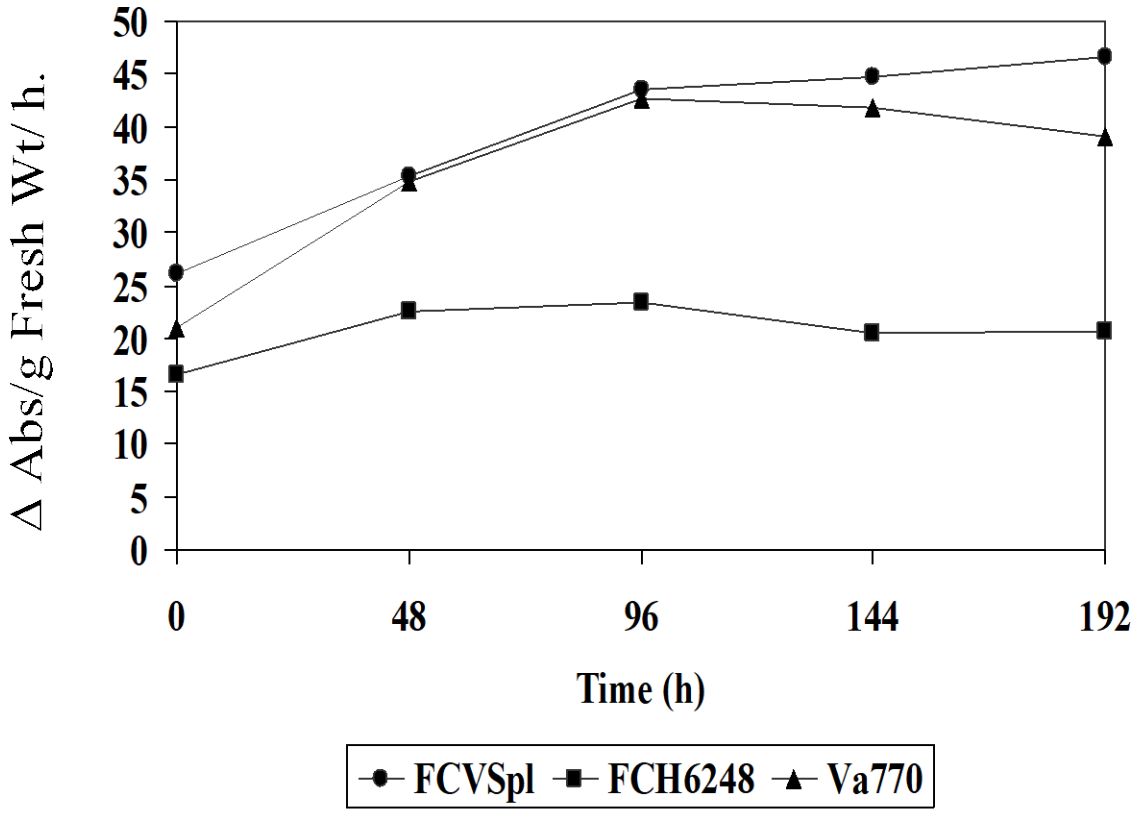


Fig. 4.5.1. Kinetics of ribonuclease activity during TMV infection *in N. tabacum* varieties

In virus susceptible variety, initial level of activity at 0 h was around 13 units in all the three treatments (Table 4.5.3). In untreated control activity remained nearly constant throughout the study of 8 days. On mock inoculation with sterile water, enzyme got activated and its level increased (55%) up to 48 h and then declined slowly. However, on TMV inoculation, steep rise in enzyme activity was recorded with in 24 h post infection. This activity remained without significant change till 144 h post inoculation.

Table 4.5.3. Effect of TMV inoculation on ribonuclease activity in *Nicotiana tabacum* cultivars

Varieties/ Time (h)	RNase activity **		
	Un inoculated control	Mock inoculated Control	TMV inoculated
Va 770 (R):			
0	12.037 ± 1.50	12.300 ± 1.04	12.150 ± 0.50
24	10.969 ± 1.92	19.250 ± 1.13	24.000 ± 0.57
48	13.904 ± 2.98	21.300 ± 1.76	25.500 ± 0.36
96	09.542 ± 2.66	24.650 ± 0.70	29.250 ± 0.05
144	14.044 ± 0.31	28.420 ± 0.43	29.120 ± 0.51
CD at P= 0.05	2.29		
FCH 6248 (T):			
0	11.989 ± 0.45	12.150 ± 0.78	11.850 ± 0.25
24	10.080 ± 1.42	17.960 ± 0.40	15.650 ± 0.46
48	10.219 ± 0.26	18.120 ± 2.06	14.960 ± 0.73
96	09.880 ± 0.51	19.560 ± 1.31	12.570 ± 0.71
144	16.550 ± 0.62	20.530 ± 0.94	14.290 ± 0.64
CD at P= 0.05	1.56		
FCV Special (S):			
0	13.158 ± 1.57	12.960 ± 0.74	12.850 ± 0.17
24	15.760 ± 1.35	19.500 ± 0.82	25.410 ± 1.05
48	13.450 ± 0.14	20.120 ± 0.77	25.410 ± 0.52
96	10.995 ± 2.21	18.250 ± 1.27	23.640 ± 0.39
144	14.525 ± 2.01	15.360 ± 0.26	24.820 ± 1.29
CD at P= 0.05	1.97		

(R) – Resistant; (T) – Tolerant; (S) - Susceptible
** Change in absorbance at 260 nm g^{-1} fresh weight h^{-1}

Eventhough mock inoculation increased the enzyme activity, TMV inoculation resulted in 30% higher values, thereby indicating the influence of viral infection on enzyme activity as can be seen from the Fig. 4.5.2.

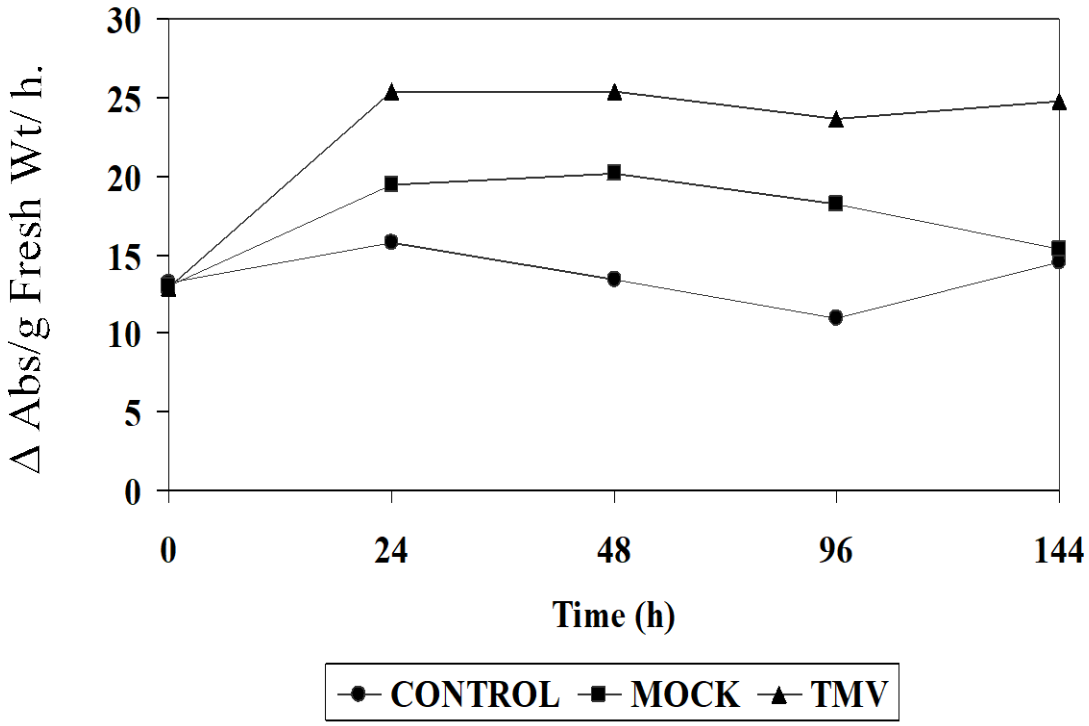


Fig. 4.5.2. Kinetics of ribonuclease activity during TMV infection in FCV Special

Initial enzyme activity was about 12 units in resistant variety. Activity remained almost at the same level in untreated control during the course of experimentation. Mechanical damage by mock inoculation activated the enzyme to an extent of 57% with in 24 h similar to that in susceptible variety. But the increase in activity continued up to 144 h post treatment and then declined. On TMV inoculation steep increase in activity (98%) was noticed with in 24 hours post-treatment. Computation of the results with and without TMV inoculation as shown in Table 4.5.4 clearly brings out the fact that viral infection triggers almost 24% higher activity initially, which decreased with time.

Table 4.5.4. Increase in ribonuclease activity with and without TMV inoculation to leaves of resistant cultivar

Time (h)	RNase activity **		
	Mock inoculated control	TMV inoculated	% Increase
0	12.300 ± 1.04	12.150 ± 0.50	--
24	19.250 ± 1.13	24.000 ± 0.57	24
48	21.300 ± 1.76	25.500 ± 0.36	20
96	24.650 ± 0.70	29.250 ± 0.05	18
144	28.420 ± 0.43	29.120 ± 0.51	15

** Change in absorbance at 260 nm g^{-1} fresh weight h^{-1}

Tolerant variety had initial ribonuclease activity of about 12 units and remained constant throughout the period of study in uninoculated plant. On mock inoculation, there occurred 48% activation with in 24 hours. Slow but gradual increase was noticed thereafter. However, tolerant variety behaved differently to viral infection than that of susceptible and resistant varieties. TMV entry activated the enzyme with in 24 hours post inoculation, and this increase was only 32% as compared to 100% increase in susceptible and resistant varieties. Another perceptible difference in the behaviour of this enzyme was noticed viz. the declining trend in enzyme activity after 24 hours. Comparison of data between mock and virus inoculated plants (Fig. 4.5.3) clearly establishes the fact that the increase noticed was only due to mechanical injury (mock inoculation) and not due to viral infection. In fact, viral infection had a negative effect on the enzyme.

It is imperative that the viral infection results in increased ribonuclease activity in susceptible and resistant varieties. In contrast, TMV infection did not influence the RNase activity in tolerant variety.

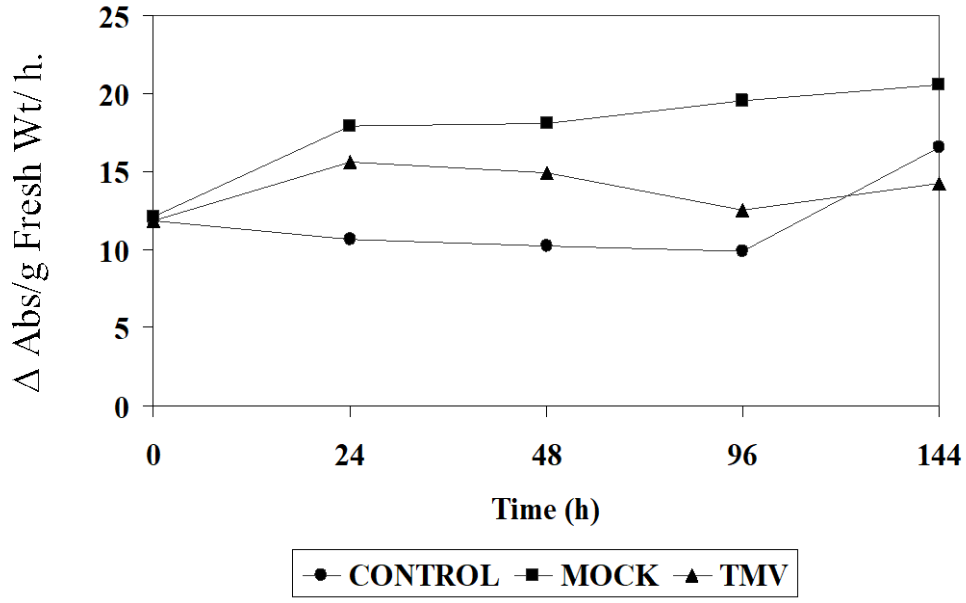


Fig. 4.5.3. Kinetics of ribonuclease activity during TMV infection in FCH 6248

4.6. Salicylic Acid

Standard graph for quantification of salicylic acid

Though, detection of salicylic acid by spectrophotometric method is feasible, minimum detection limit is 2 µg. As salicylic acid content in plant tissues was below the minimum limit of detection, a more sensitive and specific spectrofluorescence method was adopted for estimation. The excitation and emission fluorescence maxima derived for salicylic acid dissolved in methanol were at wavelengths 300 nm and 400 nm respectively, as depicted in the graph (Fig. 4.6.1)

To estimate salicylic acid using spectrofluorophotometric method, a standard graph was prepared. The values of relative intensity at excitation & emission wavelength of 300 and 400 respectively for0.2 to5.0 µg salicylic acid were recorded. The values were plotted to get the standard graph. The relative intensity values from 0 to 2.0 µg salicylic

acid followed a linear path (Fig. 4.6.2). The constants for K and B were determined as 3.2279 x 10^{-3} and 0.0513 respectively. Using these K and B values and substituting the absorbance of unknown sample in equation "concentration (µ mole) = (K x Fluorescence intensity + B)" the concentration of salicylic acid was calculated in samples.

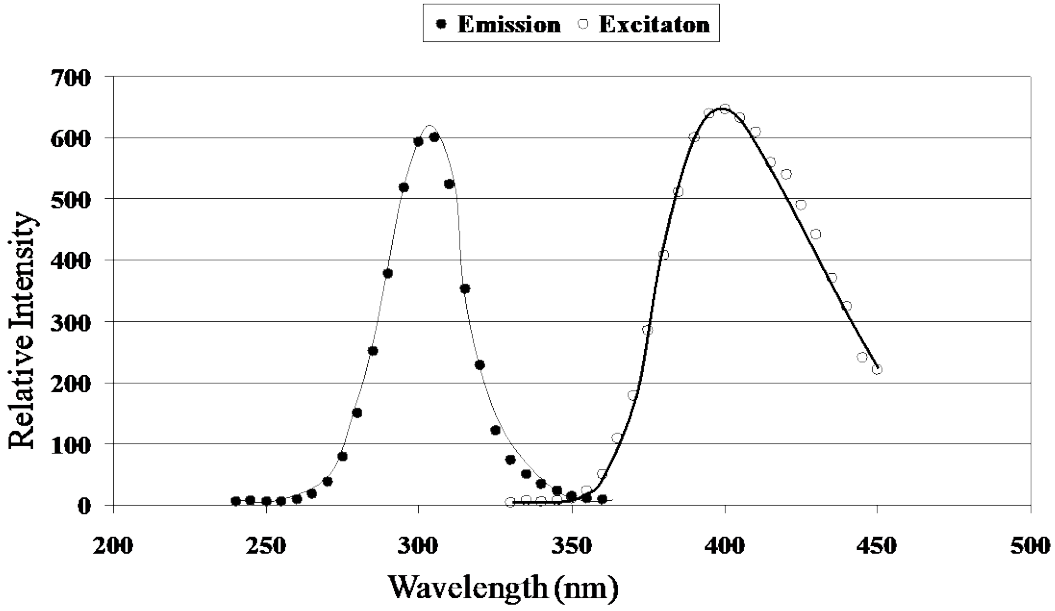


Fig. 4.6.1. Emission and absorption spectra of salicylic acid

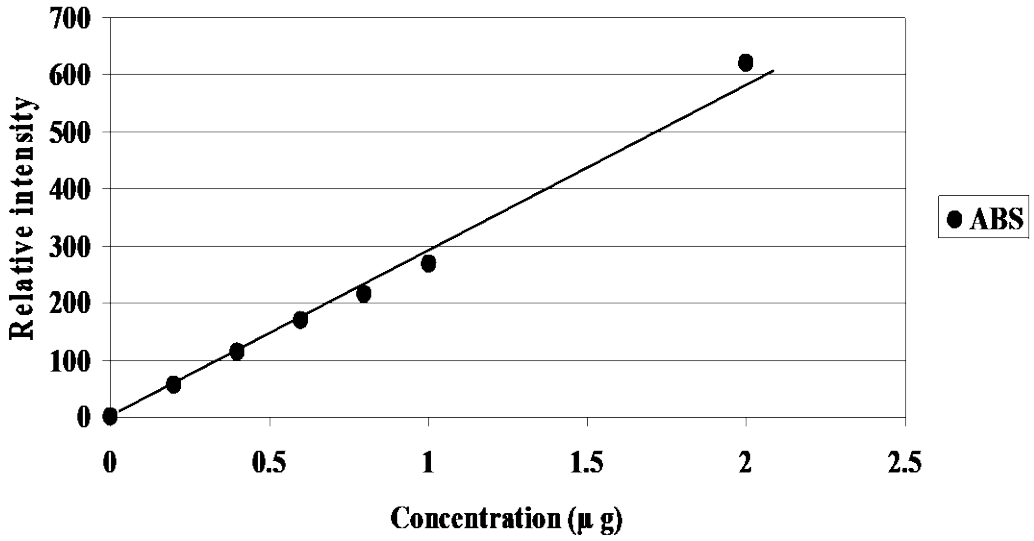


Fig. 4.6.2. Standard graph for salicylic acid estimation

Recovery of extractable salicylic acid in spiked samples

When graded concentrations of authentic salicylic acid were subjected to the extraction and purification procedure the recovery was 72.3%. Following this procedure, the recovery of extractable salicylic acid from plant was determined by spiking tissue samples with authentic salicylic acid. The recovery was found to be 39.5% for leaf and 36.3% for root (Fig. 4.6.3). These correction factors were computed to calculate the salicylic acid content in plant tissues.

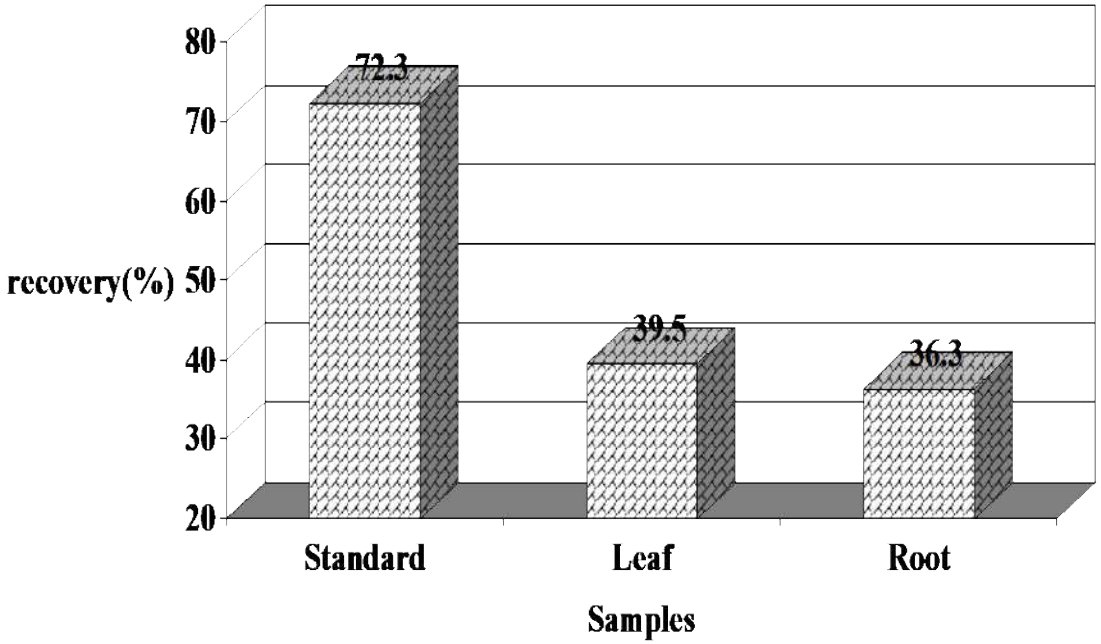


Fig. 4.6.3. Recovery rate of extractable salicylic acid in plant tissues

Effect of host - virus interaction on salicylic acid level in leaf

In susceptible (FCV Special) and resistant (Va 770) varieties salicylic acid (SA) concentration was 0.37 and 0.43 µg / g fresh weight respectively in uninoculated leaves (Table 4.6.1). The statistical analysis of the data indicates that the level of SA in FCH

6248 (tolerant variety) was significantly lower (20%) as compared to that in resistant variety. On 5th day after TMV inoculation the salicylic acid level in leaves varied among varieties. In resistant variety, 117% higher level of SA over that of uninoculated leaf was recorded. Whereas, the increase in susceptible and tolerant varieties was only 10% and 15% respectively and hence not very significant. On mock inoculation, there occurred no significant change in SA content irrespective of the variety. TMV inoculation therefore triggered over production of salicylic acid only in the resistant variety thereby suggesting a positive role for SA in the resistance mechanism.

Table 4.6.1. Salicylic acid levels in leaves of different *Nicotiana tabacum* cultivars on TMV inoculation

Variety	Salicylic acid **		
	Un inoculated control	Mock inoculated control	TMV inoculated
FCV Special (S)	0.3721 ± 0.03	0.3550 ± 0.05	0.4091 ± 0.03
FCH 6248 (T)	0.3421 ± 0.02	0.2823 ± 0.04	0.3920 ± 0.07
Va.770 (R)	0.4327 ± 0.05	0.3810 ± 0.06	0.9401 ± 0.15
CD at P= 0.05	0.11		

(R) – Resistant; (T) – Tolerant; (S) - Susceptible
** µg g^{-1} fresh weight

Effect of host-virus interaction on salicylic acid level in root

Literature study revealed that SA acts as a signal molecule to induce systemic acquired resistance in plants. Therefore, the level of SA in root was estimated because there was no report so far on the occurrence of SA in roots. Salicylic acid content in roots of uninoculated plant was about 1/ug per gram in all the three varieties and was higher as compared to leaves (Table 4.6.2). This is the first report on the presence of SA in tobacco

roots. Interestingly, on TMV inoculation there was significant reduction in SA level, in all the varieties. In susceptible and tolerant varieties, there was about 50% reduction in SA level, while reduction was only 37% in resistant variety. On the contrary, mock inoculation was found to elevate the SA level in all the three varieties. The differences were significant in tolerant (22%) as well as resistant (59%) varieties. Comparison of SA levels in roots and leaves after TMV inoculation shows significant increase in SA level in leaves, whereas the level decreased in roots. This indicates movement of SA from roots probably to leaves - the infection site.

Table 4.6.2. Salicylic acid levels in roots of different *Nicotiana tabacum* cultivars on TMV inoculation

Variety	Salicylic acid **		
	Un inoculated control	Mock inoculated control	TMV inoculated
FCV Special (S)	1.1779 ± 0.03	1.2828 ± 0.14	0.5616 ± 0.38
FCH 6248 (T)	1.1848 ± 0.14	1.4413 ± 0.41	0.5184 ± 0.19
Va.770 (R)	0.8299 ± 0.09	1.3198 ± 0.21	0.5244 ± 0.19
CD at P= 0.05	0.19		

(R) – Resistant; (T) – Tolerant; (S) - Susceptible

** $\mu g\ g^{-1}$ fresh weight

Salicylic acid as a biochemical marker in breeding experiments

The results described above implied that SA content in leaf could be used to forecast the disease resistance in breeding experiments. A higher level of SA in response to viral infection indicates resistance. In order to validate this hypothesis, breeding experiments were performed (Fig. 4.6.4; 4.6.5; 4.6.6 and 4.6.7). The design of the experiment is given below.

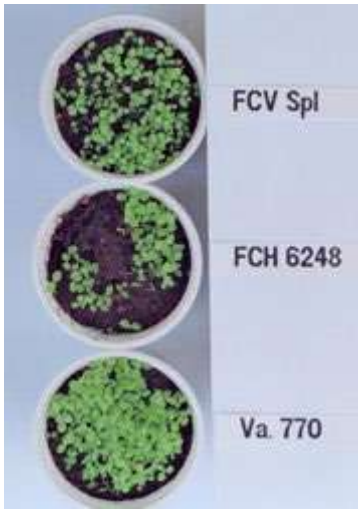


Fig. 4.6.4. Seedlings of parents raised for the study.

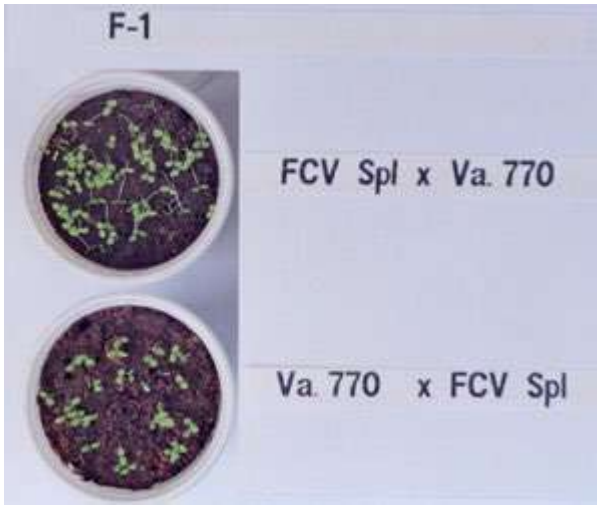


Fig. 4.6.5. Seedlings of F_1 generation derived from breeding experiments.

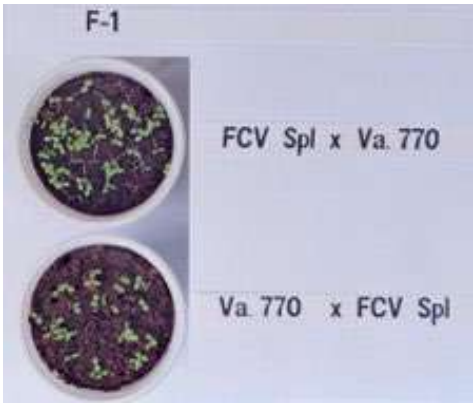


Fig. 4.6.6. Pot culture seedlings ofF_2 generation derived by selfing F_1 derivatives of the cross.

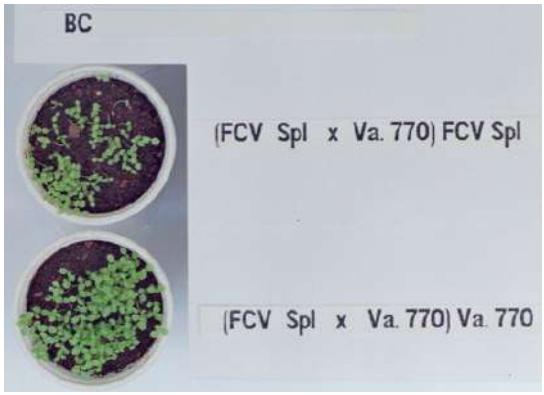
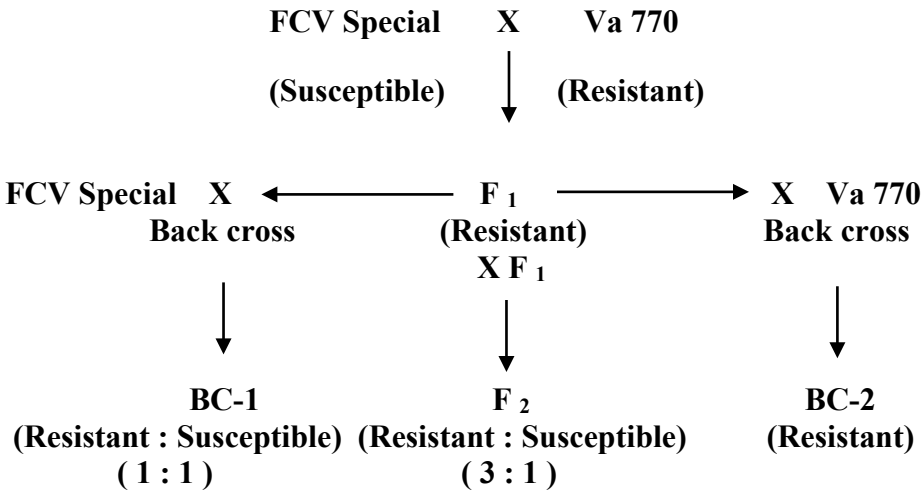


Fig. 4.6.7. Seedlings of back cross derivatives raised in cups.



Theoretically when the susceptible variety is crossed with resistant variety all the F_1 progenies should be resistant as per Mendalian law of inheritance, because resistant gene is dominant over the susceptible. And when they are allowed to self-pollinate, in the F_2 progeny the genes will segregate to produce resistant and susceptible plant in the ratio of 3:1. The F_1s when backcrossed with resistant parent, all the resulting progenies BC-2 will be resistant to TMV infection. On the other hand, if F_1 is backcrossed with susceptible parent, the progenies BC-1 will show segregation of resistant and susceptible plants in the ratio of 1:1. Accordingly, higher level of SA is expected in all the F_1 progenies as well as in the progenies derived from backcrossing with resistant parent. However, progenies resulting from F_1 backcrossed with susceptible parent should have higher level of SA in only 50% of the population. The results of SA content are given in Table 4.6.3. The values were found as per prediction in all the progenies. When the F_1 was selfed, 75% of the population had increased levels of SA. This shows that the SA content in the progeny also followed the same segregation pattern of typical Mendelian ratio of 3:1. The ratio of resistant : susceptible segregates obtained both by SA estimation on day 3 (Fig. 4.6.8) and by identifying plants based on physical appearance of necrotic local lesions on day 5 after TMV inoculation were comparable (Table 4.6.4**)**. In addition,

the results also indicate that the estimation of SA can also be used to forecast TMV disease resistance in breeding experiments.

Table 4.6.3. Salicylic acid levels in viral infected leaves of different progenies of cross in breeding experiment

Generation	Description	SA µg /g fresh wt.)**
FCV Special	Parent – 1 (P1) (Susceptible)	0.3582 ± 0.02
Va 770	Parent – 2 (P2) (Resistant)	0.9360 ± 0.02
F_1	P1 crossed with P2 (Resistant)	0.8618 ± 0.05
F_2	F1 selfed (Susceptible)	0.3649 ± 0.04
F_2	F1 selfed (Resistant)	0.8980 ± 0.01
BC	F1 backcrossed with P1 (Susceptible)	0.3652 ± 0.02
BC	F1 backcrossed with P1 (Resistant)	0.8877 ± 0.01
BC	F1 backcrossed with P2 (Resistant)	0.8634 ± 0.05
CD at P=0.05		0.0385

**SA content at 3 days after TMV inoculation

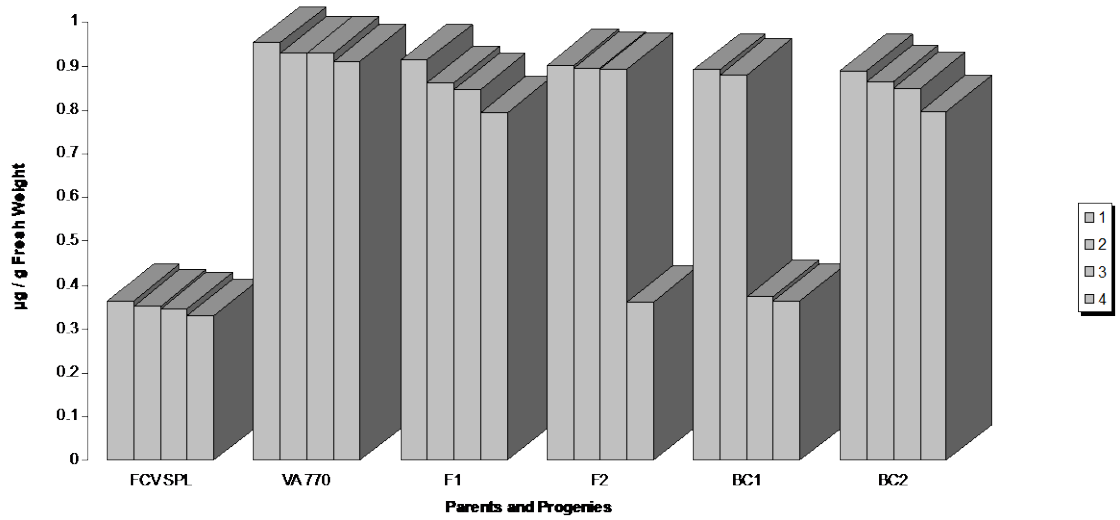


Fig. 4.6.8. Salicylic acid levels in virus infected leaves in different generations of breeding experiment

Table 4.6.4. Mode of inheritance in F_1, F_2 and BC generations in FCV Special x Va 770 cross for tobacco mosaic virus disease resistance

Parents and Crosses	Code**	Resistance frequency		Susceptiblity frequency		X^2
		Observed	Estimated	Observed	Estimated	
FCV Special	S	0	0	50	50	-
Va 770	R	40	40	0	0	-
F_1	S x R	40	40	0	0	-
F_2	S x R	31	30	09	10	0.13*
BC-1	(S x R)S	19	20	21	20	0.10*
BC-2	(S x R)R	40	40	0	0	-

** S= Susceptible, R= Resistant. * Highly acceptablechi-square value (<p=0.05)

4.7. Protein Band Profile in Seed

Oil content of seeds of different varieties in the present study

Data of oil content as determined by Soxhlet extraction of seeds with hexane is given in the Fig. 4.7.1. Oil content in seeds ranged from 32% to 43% among the varieties tested. There was no definite trend of variation among the varieties based on the reaction their reaction to TMV infection.

Seed protein profile

Protein profile of seeds as revealed by polyacrylamide gel electrophoresis after staining is given as plate 1 & 2. Coomassie blue staining pattern indicates 4 major protein bands. There is no significant difference among the varieties (Fig. 4.7.2). In order to visualize the minor bands, high sensitive silver staining was performed. Eventhough

several minor bands were visualized; there was no difference in the band counts between the varieties (Fig. 4.7.3). However, there occurred differential intensities of staining in some bands.

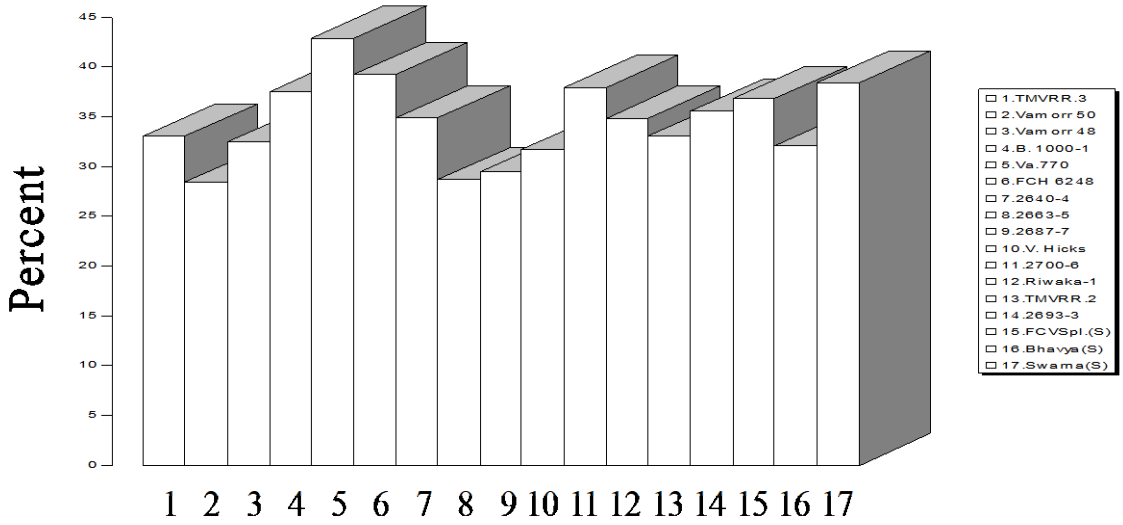


Fig. 4.7.1. Seed oil content of different genotypes

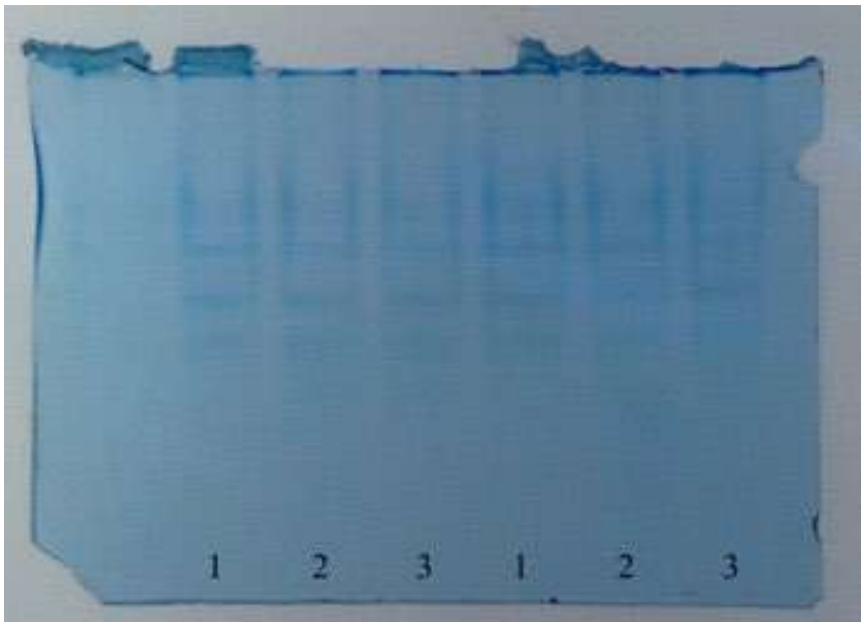


Fig. 4.7.2. Coomassie blue stained SDS polyacrylamide gel showing seed protein band profile.(1. Va 770; 2. FCH 6248; 3. FCV Special).

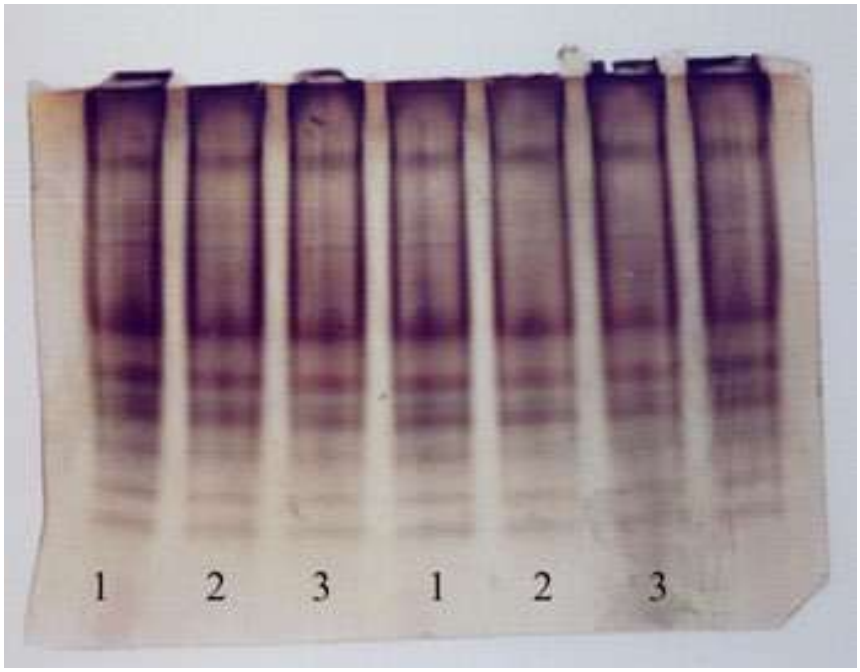


Fig. 4.7.3. Seed protein band profile of different varieties in polyacrylamide gel as visualised by high sensitive silver staining. (1. Va 770; 2. FCH 6248; 3. FCV Special).

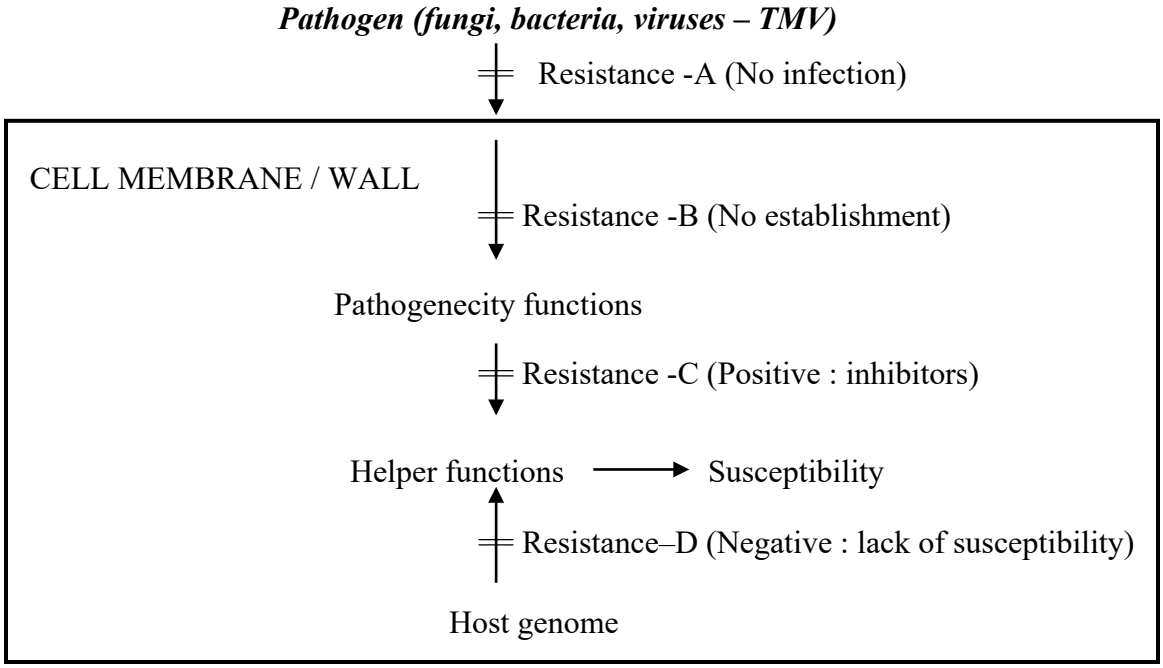
5. Discussion

5. DISCUSSION

Plants like any other biological system live in the environment, which offers many types of stresses. Since the higher plants lack locomotion, they cannot keep themselves away from the source of stress. Living organisms are subjected to physical, chemical and biological stresses. Temperature extremities, flooding, drought, injury etc., are the physical stresses. Whereas, the chemical stresses include, salinity, heavy metal toxicity, pesticides etc. The most important biological stress is infection by pathogen viz., fungi, bacteria and viruses. In addition, senescence, growth and other biological stresses are also of importance. In order to compensate their inability to retreat from the source of stress, plants have developed different mechanisms to protect against the stress.

Proposed model for the management of pathogen induced stress by plants

A predictive model describing the stress management by higher plants during pathogenesis as proposed by Fraser (1994) is shown below.



When the pathogen comes in contact with the host the first step of stress management begins. This may be described as 'Resistance-A', wherein the pathogen fails to make an entry into the host cell itself. Thick cuticle or dead bark tissues or dry plant surface may themselves act as barriers for the entry of the pathogen.

On the other hand, if the pathogen succeeds in getting into the host cell, the host triggers the second step of stress management wherein, a cascade of biochemical alterations take place to prevent the establishment and spread of the pathogen. This is termed 'Resistance-B', which is the first intracellular event. Polyphenol oxidase, phenylalanine ammonia-lyase, lipoxygenase and peroxidase are some of the enzymes involved in this mechanism. The levels of these enzymes are known to increase during this event. In case of TMV infection in tobacco this event culminates in the formation of necrotic lesion.

In plants during infection by virus, viral genome is released into the host cell to multiply itself. At this juncture, as a third step of stress management 'Resistance C' will be induced. Here salicylic acid, which is a product from phenyl propanoid pathway, is known to play a role in offering the resistance through the induction of specific proteins which prevent viral multiplication / pathogenesis. Accumulation of salicylic acid is noticed in the virus-infected cell and the local increase induces the formation of a certain species of proteins called 'Pathogenesis Related Proteins' (PR-Proteins). This is called positive resistance, viz., the host positively produce a factor (PR- Protein), which prevents multiplication of the pathogen which in turn results in resistance.

The host may also resist pathogen multiplication by indirect mechanism, wherein the host cell does not provide helper functions for viral synthesis. This is termed 'Resistance- D', wherein, ribonuclease (RNase) plays a pivotal role. Virus multiplication precedes hydrolysis of the host RNA catalyzing RNase to generate purines, pyrimidines, phosphate and ribose, which are used for the synthesis of viral RNA. Hence, level of RNase regulates viral multiplication. If the repression of the RNase is triggered by the host, the intracellular RNase level in the infected site gets depleted. This leads to failure of the accumulation of building blocks (helper function) for the synthesis of viral RNA and viral protein. This chain of reaction due to repression of RNase ultimately prevents *de novo* synthesis, multiplication and spread of virus. As a result, the plant becomes tolerant / resistant to the pathogen. In this type of 'Resistance D' initially multiplication of virus takes place due to the availability of "housekeeping" RNase providing building blocks for the initial *de novo* synthesis of the virus to a limited extent. However, repression of the enzyme takes place due to infection which does not allow further multiplication of virus, due to lack of providing helper functions.

The foregoing analysis clearly brings up the fact that the plant becomes susceptible, if it allows the entry and multiplication of the virus. On the other hand, if the host does not allow the entry of the virus or permit its entry but does not allow the virus to multiply and spread, the plant becomes resistant. The tolerant plant is the one, which allows the entry and allows initial intracellular multiplication but prevents further multiplication.

TMV infection process

In tobacco damage caused by TMV infection is of great economic importance. The interaction between host and pathogen requires prior recognition and acceptance leading to susceptibility or recognition and rejection resulting in resistance. In either case the host system exhibits several biochemical changes leading to well defined symptom development.

Mechanism of susceptibility

Tobacco mosaic virus infection leads to a characteristic symptom of mottling and blotched pattern of light and dark green areas on leaf (Fig. 5.1 & 5.2) giving a look of mosaic of colours. The virus initially develops these symptoms (Fig. 5.3 & 5.4) in the infected leaf. As the virus moves systemically in plant the subsequently formed leaves also develop the mosaic symptoms. The light green (chlorotic) tissues contain less chloroplast, thus causing photosynthetic deficiency resulting in reduced growth of plant (Fig. 5.5). In case of severe infections, plants are usually stunted and lamina of the leaf is considerably reduced in size giving a filiform (ribbon like) appearance (Fig. 5.6). Yield and cured leaf quality are greatly affected due to reduced growth and disease symptoms.

Mechanism of resistance

In order to manage the infection several studies were carried out to identify the source of resistance. The wild genotype *Nicotiana glutinosa* was found to be resistant to TMV infection. When TMV infects *N. glutinosa,* necrotic spots called primary or local lesions are produced on the leaf (Fig. 5.7). Necrotic lesions result from rapid death of infected cells; as a result, TMV gets localized in a small area. This necrotic response is

Fig. 5.1. Tobacco leaves of susceptible variety showing initial symptoms of chlorosis and vein clearing due to TMV infection.

Fig. 5.2. Advanced TMV susceptible symptoms of light and dark green mosaic patches on tobacco leaf.

Fig. 5.3. Susceptible variety showing mechanical injury on leaf at 5 days after TMV inoculation.

Fig. 5.4. TMV susceptible plants showing disease symptoms on leaf at 3 weeks after TMV inoculation. (0-Uninoculated; 1-Inoculated)

Fig. 5.5. Susceptible plant showing reduced growth on TMV infection.

Fig. 5.6. Different symptoms of susceptibility on plants. (1. Healthy leaves; 2. Mottled leaves; 3. Filiform leaves)

Fig. 5.7. Leaf of resistant variety showing necrotic lesions developed at 5 days after TMV inoculation.

Fig. 5.8. Healthy looking plant of resistant variety Va 770 at 3 weeks after TMV inoculation. (0 –Uninoculated; 1 – Inoculated)

attributed to hypersensitivity; the rate of TMV multiplication is slow, while the cell death is rapid, leading to limited spread of the virus. *N. glutinosa* is the source of resistance in all the TMV resistant commercial tobacco cultivars including Va 770 used in the present study (Fig. 5.8). The associated quality defects limit the usefulness of this variety Va 770 in resistance breeding of high quality conscious crop - flue cured tobacco.

Mechanism of tolerance

In tolerant variety FCH 6248 used in the present study, the plants initially show the mild susceptible symptoms of mottling and chlorosis giving mosaic appearance (Fig. 5.9 & 5.10). The leaves do not get distorted and even the mild symptoms disappear as the growth progresses, unlike that of susceptible variety (Fig. 5.11, 5.12, 5.13, 5.14). The plants thus do not suffer either in growth or yield causing no economic loss. As on now the tolerance can be identified only by the symptom disappearance and lack of disease severity during the plant growth but not at early stages.

Importance of tolerance in tobacco breeding

Most of the biochemical approaches in tobacco-TMV system are restricted to identification / selection of resistant variety. However, importance of other qualities needed for commercial purposes are not considered in the breeding programmes aimed at deriving resistant variety. Pathogen resistant progenies in tobacco invariably lack desired manufacturing qualities due to linkage of resistant genes with undesired commercial qualities (Lewis *et al*., 2007). Though there is no report of virus developing resistance to this vertical resistance mechanism, there is always a risk of the hypersensitive expression getting suppressed at temperature above 28ºC as described by Kassanis (1952).

Fig. 5.9. TMV tolerant variety FCH 6248 showing mechanical injury at 5 days after virus inoculation

Fig. 5.10. Mild disease symptom development in TMV tolerant variety FCH 6248 at 3 weeks after TMV inoculation. (0 –Uninoculated; 1 – Inoculated)

Fig. 5.11. TMV susceptible variety FCV Special showing leaf malformation at 3 weeks after virus inoculation.

Fig. 5.12. TMV tolerant variety FCH 6248 showing only mild chlorotic symptoms at 3 weeks after virus inoculation.

Fig. 5.13. TMV susceptible variety showing advanced symptoms of disease at 4 weeks after virus inoculation.

Fig. 5.14. TMV tolerant variety showing only low intensity symptoms ofdisease at 4 weeks after virus inoculation.

Tolerant variety is the solution for the farming community because, it is this tolerant variety, which has the desired economic qualities and at the same time prevents or restricts the invasion of the pathogen. In addition, the tolerance character is not vertical resistance as that of glutinosa type of resistance. Hence, TMV is less likely to develop a virulent strain to break the barrier. In the earlier research at our Institute we have developed a tolerant cultivar starting with a susceptible parent. This tolerant variety is recognized as FCH 6248. Field studies of this variety revealed that it lacks certain manufacturing qualities although has many other desirable traits required in cultivation and trade. Because of the few undesirable qualities, mass cultivation of FCH 6248 could not be undertaken. The future plan of the Institute, where the author is working includes breeding programme to improve the tolerant variety FCH 6248 by bringing in the required 'manufacturing qualities'.

The objective of the present study is to identify biochemical tools for the rapid detection of tolerant recombinants to aid in the selection and identification of the required phenotype early in breeding experiments. Such a search has resulted in fixing RNase as a possible biochemical marker to identify tolerant recombinants.

5.1. Peroxidase

The role of peroxidase in plant resistance has been attributed to its ability to oxidize important metabolites either of the parasite or of the host plant.

Absence of peroxidase activity in seed as observed in the present study supports the observation reported earlier by Sheen (1969). The enzyme was activated only during germination to carry out the physiological functions required for active growth of

seedling. All the varieties behaved similar in enzyme activation process, except for the plateauing of enzyme level at 12 days in susceptible variety.

Peroxidase activity varied with age of leaf. The bottom senescing leaves had 11 to 32% higher activity than that in top growing leaves. Similar trend was reported by Lovrekovich *et al*. (1968), Sheen (1969), Jong (1972) and van Loon (1976). The increase in peroxidase activity in old senescing leaves is attributed (Sheen, 1969) to the induction evoked by the direct contact of substrates with enzymes after the rupture of cell membrane due to ageing. The old leaves in tolerant variety showed highest activity, possibly because of faster senescence of leaves in the variety as compared to the other two.

Viral inoculation elicited peroxidase enzyme significantly, only in resistant variety and neither in susceptible nor in tolerant plants. The activity burst is more pronounced in *N. glutinosa,* a wild species that was the donor of TMV resistance in the resistant variety. Increase in peroxidase activity due to hypersensitive reaction in resistant variety was similar to the findings of Solymosy *et al*. (1967), Novacky and Hampton (1968), Simmons and Ross (1970, 1971) and Weststeijn (1976). As explained by Jong (1972), peroxidase is probably involved in channeling the oxidizing power of hydrogen peroxide to some more useful purpose like, lignification and IAA oxidation. The ability of peroxidase to oxidize phenols is related to the induced changes of the enzyme in resistant leaves as a response to inoculation (Simmons and Ross, 1971). Structural disruption brought about due to infection, presumably would facilitate the mutual availability of peroxidase and its substrates.

The inconsistency observed in the present study in grownup plants, is possibly because they are subjected to several environmental interactions. The dual stress (mechanical and biochemical) probably has an additive effect, increasing complexity to draw no definite correlations between viral interaction and enzyme activation. As explained by Daly and coworkers (1970), high peroxidase activity may be the result rather than a cause of incompatibility. Even though peroxidase activity is triggered due to virus infection, the response is not specific and hence perhaps cannot be used as an exclusive marker to aid selection in breeding programmes.

5.2. Polyphenol Oxidase

The presence of phenolic compounds in plants, their oxidation following injury, either mechanical or due to infection and relatively high toxicity of the oxidation products prompted to ascribe a role to polyphenol oxidase in disease resistance.

Polyphenol oxidase activity was not detected in seeds and up to 2 days during germination. Sheen (1969) also reported absence of enzyme activity in seeds thereby supporting the present study. Extremely low level of enzyme activity during germination was observed in tolerant variety, while; it was highest in the susceptible variety from 10^{th} day of germination. Faster growth of seedlings in susceptible variety and concurrent need for higher energy demands might be the possible reason for increased polyphenol oxidase activity recorded in the present study (Jong, 1972).

The findings clearly show higher enzyme activity in young and expanding leaves than old senescing ones in all the varieties. This is in agreement with the results obtained by Jong (1972) and van Loon (1976). The high enzyme activity in immature leaves and

its gradual decline in association with maturation were correlated with higher energy demand in young growing leaves (Jong, 1972). Jong also ascribed control of redox stress of polyphenols within the photosynthetic cells of leaf as a plausible reason for enhanced activity.

Polyphenol oxidase activation level in tolerant and resistant varieties on TMV inoculation was not significant as compared to mock inoculation causing only mechanical injury. The level of activation was comparatively higher in susceptible variety. Hence, role of polyphenol oxidase enzyme in resistance mechanism is not specific. As explained by Mayer and Harel (1979), the results don't rule out the function of polyphenol oxidase enzyme in disease resistance though no experimental proof was obtained in the present study. However, polyphenol oxidase enzyme may not be useful as a marker in screening genotypes for TMV resistance.

5.3. Lipoxygenase

Lipoxygenase (LOX) enzyme initially gained commercial importance because of its property to alter the quality of foodstuffs during long-term storage. Initiation of a series of physiological processes during host pathogen interaction by the catalytic function of LOX in plants is of importance in the present investigation.

When LOX activity in seeds was measured, significant variations among varieties were recorded. Varieties having resistance as well as tolerance to tobacco mosaic virus showed around 2 times higher LOX activity than that in susceptible variety. Tobacco seed is known to contain more than 40% oil. Maestri and Guzman (1993) have reported predominance of linoleic acid constituting more than 70% of total lipids in tobacco seed

oil. Linoleic acid being a good substrate in LOX pathway, the enzyme may use linoleic acid leading to the production of compounds like jasmonates (Koda, 1992). Farmer and Ryan (1992); Creelman *et al*. (1992) have attributed jasmonates to carryout signal transduction in plants on wounding and pathogen attack. Tobacco seeds are also known to contain many defense-related proteins to counter the attack by pathogens (Bowels, 1990). Hence, higher level of LOX in TMV resistant variety may be indicative of its incompatible interaction with virus.

To confirm this hypothesis, LOX activity level in seeds of several other genotypes both susceptible and resistant to TMV were measured. All the resistant varieties recorded higher LOX activity than that of susceptible. The activity in resistant varieties ranged from 0.86 to 2.07 units (Fig. 4.3.1), while in susceptible varieties it was in the range of 0.40 to 0.68 units. These results are in agreement with the findings of Nagarathna *et al*. (1992) wherein, higher activity in sorghum seeds was positively correlated to the increase in powdery mildew resistance level of a variety. Hence, level of LOX activity in seed may serve as a marker to identify resistant genotypes. However, in breeding programmes the biochemical marker should be relevant not only to seeds but also to seedlings to enable generation advancement.

Infection of TMV at seedling stage causes high economic losses. To identify the difference in enzyme activity in resistant variety if any as an indicator, the activity level during seed germination was studied. As reported in a number of plant species, increase in LOX activity during germination and seedling growth was recorded in three varieties of tobacco. The activity reached a peak on day 12 and then declined. Though such a trend was reported in other plants, number of days taken to reach activity peak varied with the

crop (Siedow, 1991). The activity level up to day 4 and beyond day 14 were higher in resistant variety over the other two. Possibly, this trend indicates the protective role of LOX to fight off pathogen. Day 2 is highly vulnerable stage, because tissue wounding is possible during sprouting and that may serve as the site of infection to pathogens including TMV. The higher activity level in seedling beyond 14 days in resistant variety may help seedlings to ward off TMV infection. It is interesting to note (Table 4.3.2) that in susceptible variety also increased level of LOX was recorded from day 4 to day 12. This increase is because of the faster seedling growth in variety FCV Special during early stages of growth (Fig. 5.3.1).

LOX hydrolyses lipids in the membranes of the storage cells thereby enhancing their permeability and subsequently facilitating the movement of large amounts of nutrients to the growing embryo. This observation supports the finding of Vick & Zimmerman (1987).

A remarkable difference in LOX activity among the varieties when challenged with TMV clearly indicates the role of the enzyme in disease resistance. A spurt in LOX activity by 24 hours post inoculation was recorded only in resistant variety. The results in the present varieties under study are in agreement with the findings of Kunstler *et al*. (2007), who attributed the early and greater induction of LOX activity to TMV disease resistance.Though, wounding of leaf also activated the enzyme the intensity was significantly low. Ruzieska *et al*. (1983) reported increase in LOX activity on TMV infection parallel to hypersensitive reaction in resistant genotype Xanthi nc. Lipoxygenase catalyses hydroperoxidation of fatty acids and their decomposition products stimulate breakdown of cellular membranes leading to localized cell death

FCV Special FCH 6248 Va 770 *N. glutinosa*

Fig. 5.3.1. Plants of different genotypes used in the study showing variation in plant morphology and growth rate.

commonly associated with hypersensitive reaction (Siedow, 1991). Methyl jasmonate, a volatile fatty acid derived compound, was shown to induce proteinase inhibitors in tobacco through possible signaling pathway (Farmer & Ryan, 1990). The findings of the experimentation in tobacco cell culture by Anderson *et al*. (1998) prompted them to suggest that the induction of lipid peroxidation by a salicylic acid radical may be involved in the action of SA and that one or more of lipid peroxidation products may mediate signal transduction leading to pathogenesis related protein expression. These findings strongly support the possible use of lipoxygenase activation on TMV infection as a biochemical marker in the identification of resistant genotypes in the breeding programme. However, LOX activity measurement in seeds can be a useful marker to sort out resistant genotypes from that of the susceptible ones.

5.4. Phenylalanine Ammonia-Lyase

In tobacco, incompatible interaction with TMV infection is through hypersensitive local lesion reaction to prevent further viral spread. Activation of phenylpropanoid metabolism during lesion development leading to cell death is one of the most studied phenomena and is believed to result in phenolic polymer deposition, which builds up new mechanical barriers against pathogen invasion (Favali *et al*., 1978; Legrand, 1983). The enzyme phenylalanine ammonia-lyase (PAL) primarily regulates this pathway. PAL catalyses the deamination of L-phenylalanine to yield trans-cinnamic acid and ammonia. Cinnamic acid is the precursor of all phenylpropanoids (Hahlbrock and Scheel, 1989).

In the present study, PAL activity in leaf of resistant variety (Va 770) was elicited by 12 hours post TMV inoculation and the level was two fold higher than in mock-inoculated leaves. Higher enzyme induction on virus infection is attributed to the incompatible interaction leading to hypersensitive reaction.

The time course studies of enzyme activity in resistant, tolerant and susceptible cultivars at seedling stage clearly indicated that the elicitation of enzyme was restricted to resistant plants. A spurt in the enzyme activity was recorded within 48 h post inoculation, i.e. prior to appearance of necrosis. Though, the enzyme was activated in susceptible cultivar due to TMV inoculation, the intensity was comparable to that induced due to mock inoculation, without appreciable increase. This is in confirmation with the results obtained by Pellegrini *et al*. (1994), Legrand *et al*. (1976) and Fritig *et al*. (1973). Inhibition of PAL activity was reported to increase lesion size by two to four folds and also detection of virus particles beyond lesion boundary (Massala *et al*., 1980) indicating direct role of the enzyme in limiting virus spread. Fritig *et al*. 1972 reported that the two phenomina i.e., increase in phenolic compound biosynthesis and formation of necrotic local lesions occur at the same time in the course of the hypersensitive reaction. Because, necrosis and the stimulation of phenylpropanoid pathway are close and parallel in time as well as amplitude, PAL the first enzyme regulating this pathway appear to be good biochemical marker of the necrotic reaction. The experiments performed in the present study indicate that elevation of PAL activity can be a good biochemical tool to detect resistant progeny. However, the tolerant variety did not show marked activation of PAL on TMV inoculation in the present study. Likewise, the enzyme activity compared with susceptible variety. These results clearly reveal that PAL is not a useful marker to

identify TMV tolerance of FCH 6248 and hence this enzyme has limited use as a marker in the present tolerant host-virus interaction system.

It is also to be noted that PAL is the primary enzyme in the biosynthesis of salicylic acid, which is implicated in signal transduction pathway leading to systemic acquired resistance.

5.5. Salicylic Acid

Salicylic acid (SA) was initially known only for its therapeutic property on human ailments. Its role in plant as an endogenous regulator of flowering has gained importance with the identification of its presence in thermogenic plants (Raskin *et al*., 1987). White (1979) observed that exogenously applied SA (aspirin) induced resistance to TMV in tobacco. Demonstration of SA accumulation at the sight of necrosis following infection in tobacco plants by Malamy *et al*. (1990) and Metraux *et al*. (1990) have given a new dimension to the role of SA in resistance.

In the present study, a comparative estimation of SA in TMV resistant, tolerant and susceptible varieties were made to explore the possibility of its use as a marker to distinguish differential response of genotypes to TMV infection.

Recovery rate of SA with the method adopted for extraction, purification and detection was 39.5% in leaf and 36.3% in root, which falls within the broad range of 34 to 62 % reported by Yalpani *et al*. (1993 b). Hence, mean values of triplicate assays after correction for recovery during extraction were used for estimation.

Salicylic acid in leaves

Salicylic acid estimation in leaves indicated that there was no difference in the basal level among varieties. However, only resistant but neither susceptible nor tolerant variety showed significant accumulation of SA 5 days after TMV inoculation. In resistant variety increase in SA was 117% above the levels found in leaves of healthy plants i.e., from 0.43 to 0.94 µg / g fresh weight. This increase is in agreement with the results of Yalpani *et al*. (1991). These authors have shown that SA formation / accumulation results in the induction of PR proteins, which in turn ward off the infection. In their experiment, even 0.054 µg / g tissue of the plant was sufficient enough for the induction of PR proteins. We found in the present study 0.94 µg SA, which is about 17 times higher than the level required for PR protein induction. Hence, this must have enabled production of abundant PR proteins leading to resistance. Our studies again reveal that SA has a role in establishing resistance to TMV infection in tobacco.

Salicylic acid in roots

The basal levels of SA in root tissues of the three varieties were 2 – 3 times higher when compared to the corresponding levels in leaf tissues of the same set of plants. Probably roots either act as reservoirs of SA in tobacco or major site of SA synthesis. Interestingly, there was a drastic decline in root SA level (37-56%) in all the varieties at 5 days after TMV inoculation on leaves. The decline in SA level was not seen on mock inoculation. Viral invasion induced UDP-glucose : salicylic acid 3-*O*-glucosyl transferase enzyme activity (Enyedi and Raskin, 1993) which catalysed the formation of β -O-D-glucosylsalicylic acid. This conjugate was stored in the root. The conjugate went

undetected as the procedures of extraction adopted in the study enabled elution of SA alone and not the conjugate.

Inoculations of the three varieties lead to the enhanced SA level only in resistant variety but not in susceptible and tolerant varieties. This is because as revealed earlier, TMV inoculation increases PAL enzyme level only in resistant variety and not in the other two. This enhanced PAL activity leads to excess SA formation and thereby explains the elevated quantity of SA in resistant variety even though conjugation of SA with glucose takes place at the same level in the leaves of all the three varieties.

With strong evidence from noninvasive, *in vivo* labeling of SA with $^{18}O_2$ supported by ^{14}C-labeled studies in tobacco mosaic virus resistant Xanthi-nc tobacco, Shulaev *et al*. (1995) proposed that salicylic acid is the endogenous signal in the development of systemic acquired resistance through induction of PR proteins. The hypothesis of a novel salycylhydroxamic acid sensitive mechanism (SHAM) a sensitive signal transduction pathway, which is distinct from that leading to resistance to bacteria and fungi, proposed by Chisava *et al*. (1997) further supports SA specific induced resistance to TMV in tobacco. These evidences make salicylic acid a strong candidate as a useful biochemical marker to identify TMV resistant varieties in tobacco.

To validate the practical utility of this biochemical marker in breeding programme, SA levels in TMV inoculated leaves of first & second filial and back cross progenies derived from the cross FCV Special x Va 770 were estimated 3days post inoculation. Higher level of SA on TMV inoculation was detected only in resistant progenies. Plants with high and low SA levels followed the same segregation ratio as that

estimated on visual necrotic symptoms (Table 4.6.3). This proves the usefulness of the marker as a supportive biochemical evidence to make selections in breeding programme.

5.6. Ribonuclease

Ribonucleases (RNase) mediate the hydrolytic breakdown of ribonucleic acid (RNA) to enable their turnover in cells. Hence, RNase plays a pivotal role in a number of regulatory and developmental processes in which RNA molecules are involved. Tobacco mosaic virus obtains ribonucleic acid, the genetic material for their multiplication only from the host. Synthesis of TMV specific RNA is not the normal function of the host cell. Hence for replication and multiplication, the virus needs to depend either by *de novo* synthesis of purine and pyrimidine using host cell metabolism or by the degradation of host RNA. The findings of Reddi and Mauser (1965) showed virus obtains the building blocks by degrading host cell RNA for its multiplication. Thus, RNase plays a major role in TMV multiplication.

In the present study kinetics of ribonuclease in resistant, tolerant and susceptible genotypes on TMV infection were compared to understand role of enzyme in tolerant genotype.

In TMV susceptible variety quick ascent (2 fold) in ribonuclease activity in the leaf was observed by 24 h post TMV inoculation and remained constant at this high level till 144 hour. This is in confirmation with the findings of Reddy (1959) with Turkish tobacco in which TMV multiplies systemically. The kinetics of RNase activity on TMV infection follows the time course of synthesis of TMV RNA reported by Pelcher *et al*. (1972) and Hagiladi *et al*. (1975). They suggested that synthesis of viral RNA is at peak

during the 48-60 h and declines subsequently and lasts till 140 h after inoculation. The RNase activity level in leaf on TMV inoculation in the present study follows the same time course. The findings of level of RNase enzyme on TMV infection, by Uekusa *et al*. (1993) in cucumber cotyledons and Sindelar and Sindelarova (1995) in mesophyll protoplasts of *N. tabacum* cv. Samsun further support the view that increase in RNase activity correlates with the multiplication rate of TMV. The marginal increase in enzyme activity on mock inoculation was a temporary response possibly due to mechanical injury caused by carborundum while rubbing as reported in *Phaseolus vulgaris* by Diener (1961).

In resistant variety, substantial increase in RNase activity at the same level as in susceptible variety was noticed, though virus multiplication process is restricted by the initiation of hypersensitive reaction on TMV inoculation. This increase is probably due to release of preformed enzymes by the destruction of lysosome particles during mechanical injury as concluded by Pitt and Galpin (1971). The necrotic reaction might have triggered the release of enzyme at a higher level than due to mechanical injury alone.

Interestingly, the tolerant variety in which though TMV multiplies systemically, level of symptom development and viral count are low, behaved differently from that of the other two varieties in RNase activation by virus. The level of enzyme activation on TMV inoculation at 24 h was only 32% and remained constant thereafter. The present understanding is that, brake down products of host RNA by the action of ribonuclease enzyme serves as base materials for viral RNA construction (Reddi, 1963). The lower level of RNase activity in this tolerant variety is proposed to be limiting factor for TMV multiplication. The limited increase in enzyme activity may check the availability of host

RNA degradation product, resulting in slower rate of virus multiplication in the infected leaf. In a similar type of resistance earlier observed by Nolla and Roque (1933) in variety Ambalema, the mechanism of resistance to TMV is attributed to the reduced accumulation in cells and slow spread of the virus in host tissues (Chen *et al*., 1990). This tallies with the hypothesis made in the present system of host pathogen interaction in TMV tolerant variety FCH 6248. Absence of sudden spurt in RNase enzyme unlike that in susceptible variety may serve as a good biochemical marker to distinguish this tolerant variety (FCH 6248) from the susceptible. This marker-aided selection will be faster (by 20 days) than the conventional method of screening where one needs to wait for 120 days, till the plant grows to show mild symptoms.

5.7. Protein Band Profile in Seed

There was no significant variation among the genotypes in seed oil content and hence this cannot serve as a differential marker to separate out the varieties resistant to TMV infection. The profile of protein bands in seeds of different varieties also showed limited variation either in band count or in relative mobility of bands. Though the difference in relative intensity visualized in some bands can serve as makers, further detailed investigations are needed to assess their usefulness in breeding programme.

5.8. Biochemical Status of TMV Infected Tobacco Leaf

Comprehensive data of the parameters studied using susceptible, tolerant and resistant varieties of tobacco has been presented in Fig. 5.7.1. Peroxidase and polyphenol oxidase are not reliable parameters to identify tolerant varieties of tobacco at early stages of growth. Levels of lipoxygenase (LOX), phenylalanine ammonia-lyase (PAL) and

salicylic acid (SA) may be useful in identifying resistant recombinants. In resistant varieties levels of LOX, PAL, SA significantly increase in response to TMV infection. Thereby, study of these parameters can help in sorting out resistant and susceptible varieties in breeding experiments.

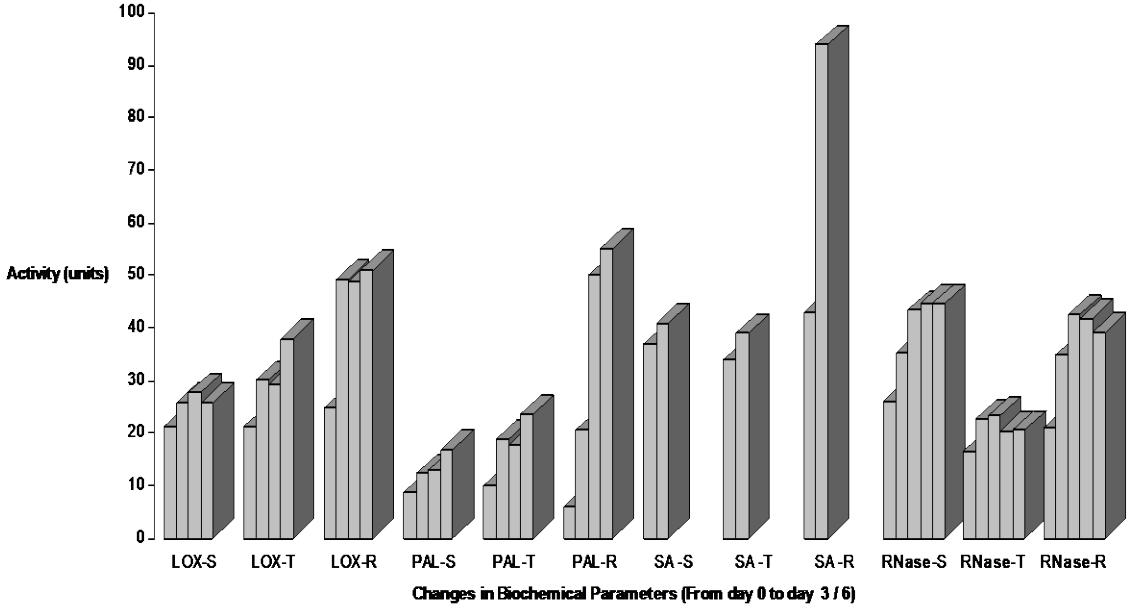


Fig. 5.7.1. Biochemical changes on TMV infection in susceptible, tolerant and resistant varieties

Interestingly the tolerant variety could be scouted out by the distinctly different profile of ribonuclease (RNase). It is to be noted that RNase activity was the lowest in the tolerant variety, which restricted virus multiplication by not providing building blocks from the host cells for the *de novo* synthesis of viral RNA. The study therefore has established the usefulness of RNase activity measurement of the seedlings to be a biochemical tool to select and identify tolerant segregants of tobacco in breeding experiments. Assay of RNase of 20 days old seedlings would greatly help in

differentiating the tolerant and susceptible recombinants. While the traditional methods take about 120 days, RNase measurement helps to sort out the progenies in as early as 20 days, thereby reducing the time (6 folds). In addition, the RNase measurement is rapid and equally reliable. The objective of the study has been successfully achieved with this findings and RNase measurement is the biochemical tool and assay of LOX, PAL and SA are complementary tests, which increase the probability of selection of tolerant variety in breeding programmes.

6. Summary and Conclusion

6. SUMMARY AND CONCLUSION

Increasing the productivity and reducing production cost are the need of the hour to sustain agriculture in general and to meet the ever-increasing demand for export oriented crops such as FCV tobacco cultivation in particular. However, diseases caused by bacteria, fungi and viruses not only take a large share out of the yield but also impair the export. Breeding for resistance to diseases using appropriate genetic resources is the best way to overcome the problem.

Flue Cured Virginia tobacco in Karnataka - a rain fed field crop of high economic significance is no exception to the pathogen attack and subsequent yield loss. Among the pathogens tobacco mosaic virus causes significant loss in yield and quality in this crop. There are no chemical control measures yet available to check the disease. Cultivation of disease resistant variety is the only way to overcome the problem. Though resistant genotypes are available, due to close linkage of resistant genes with other genes, many a times undesirable qualities especially the low-grade outturn are also transferred to the progenies along with disease resistance. This gene linkage is the limitation for effective utilization of the genetic resources in breeding programmes.

Though, the tolerant variety FCH 6248 produces better grade outturn as compared to the resistant variety, it still needs improvement to meet the requirements of the trade. This need calls for further breeding work. In traditional breeding methods, phenotypic identification of desired TMV tolerant recombinants in a large population is time consuming, labour oriented, tedious and involves risk of missing the desired recombinants. Quick and early detection methods to ensure and identify the recombinants

would greatly aid scouting and shortlisting the promising recombinants, thus help to expedite breeding programmes.

The present study was aimed at identifying biochemical markers as quick tools to detect tolerant recombinants in breeding programme. The accomplishments from the present study are summarized below.

1. An elegant pot culture system was developed to perform laboratory experiments under controlled conditions. This optimized technique enabled to grow tobacco plants aseptically in shade house in pot cultures containing acid washed sand using appropriate concentration of mineral nutrient solutions.

2. Methods were standardized to extract peroxidase, polyphenol oxidase, lipoxygenase, phenylalnine ammonia- lyase, ribonuclease and salicylic acid from laboratory grown young plants.

3. An innovative technique has been developed to separate supernatant from the lipid extracted along with the enzyme during maceration of seed. This simple technique minimized interference of the lipid in enzyme reaction, thereby enhancing consistency and reproducibility of the lipoxygenase assay.

4. Increased peroxidase activity was recorded in response to TMV inoculation only in resistant tobacco genotypes Va 770 and *Nicotiana glutinosa* but not in susceptible and tolerant varieties.

5. Kinetics of peroxidase activity showed a zigzag pattern indicating its need based role in stress management viz. infection, wounding and senescence.

6. Irrespective of the varieties, older leaves had higher peroxidase activity than the younger leaves of the same plant.

7. FCH 6248 though is a slow growing variety; the leaves mature faster than resistant and susceptible varieties.

8. This tolerant variety (FCH 6248) showed highest activity of peroxidase in older leaves thereby supporting the concept that higher activity is associated with maturity / senescence.

9. Peroxidase seems to have no role in TMV tolerance because the enzyme was elicited only during hypersensitive reaction in TMV resistant variety.

10. Inconsistency of peroxidase behaviour was attributed to the varied environmental interactions and ruled out the possibility of using peroxidase activity measurement as an exclusive marker to aid early selection in breeding programme.

11. Polyphenol oxidase and peroxidase activity were non-detectable in tobacco seeds of all varieties.

12. Interestingly highest induction of polyphenol oxidase was noticed due to TMV infection in the susceptible variety and the least in resistant variety, implying the importance of polyphenol oxidase in protecting the host against pathogen attack.

13. Mock inoculation by pricking the leaves also accelerated polyphenol oxidase production indicating the role of this enzyme in wound healing.

14. Increased formation of polyphenol oxidase in response to mock inoculation as well as due to TMV infection in susceptible variety suggests that attack by a

pathogen is considered as a stress by the host plant and therefore polyphenol oxidase is again confirmed to be a stress management enzyme.

15. Very little increase in the polyphenol oxidase level in resistant variety when challenged with TMV clearly revealed that the resistant host does not consider the attack by pathogen as a stress. This observation also explains the elevated level recorded in a susceptible variety in response to TMV infection.

16. Increased polyphenol oxidase activity at seedling stage in uninoculated susceptible variety has been explained to be associated with faster growth of the plant. This was further confirmed by recording higher activity of the enzyme in young and growing leaves of adult plant.

17. Polyphenol oxidase has been shown to be an important enzyme in tobacco plant performing a cascade of biological activities such as effecting faster growth, repair of mechanical injury, protection from infection etc.

18. Due to the multifarious functions, determination of polyphenol oxidase in seedlings may not exclusively the right parameter to identify the resistant / tolerant variety in breeding experiments.

19. Higher lipoxygenase activity in seeds of TMV resistant tobacco measured in the present study is the first report. The observation enabled to use lipoxygenase activity in seeds as a biochemical tool to screen TMV resistant genotypes. This finding is significant because of its immense value in the early detection of resistant recombinant in breeding work.

20. Enhanced enzyme activity noticed in resistant genotype at the highly vulnerable stage for infection (day 2) was indicative of the crucial role of lipoxygenase to prevent TMV infection during seed germination.

21. We propose that failure of the host plant to induce lipoxygenase in response to TMV inoculation leads to infection as supported by the low level of lipoxygenase activity recorded in the susceptible variety when challenged with TMV.

22. Lipoxygenase is also a facilitator of seedling growth. This enzyme hydrolyzes membrane lipids of storage cells to enable easy flow of nutrients.

23. Increase in lipoxygenase activity was demonstrated due to hypersensitive reaction within 2 hours post TMV inoculation in resistant variety. Higher response has proved the utility of lipoxygenase as a quick biochemical marker in identifying resistant genotypes, even before the visual symptoms of hypersensitive reaction appear.

24. Increase in phenylalanine ammonia-lyase activity by 48 hours post TMV inoculation has been proved useful as a biochemical marker to sort out resistant segregants in breeding programme.

25. Procedure for extraction of salicylic acid from leaves as well as roots and purification of salicylic acid for estimation were modified and standardized.

26. A rapid, cost effective and reliable spectrofluorescence technique with a low detection limit of 0.2 μg for salicylic acid estimation was standardized and a calibration curve was prepared to facilitate the quick quantification.

27. Usefulness of salicylic acid assay to identify resistant genotypes was confirmed. Only the resistant genotype recorded 117% higher value.

28. Presence of salicylic acid in abundance in tobacco roots is the first report. Significant decrease in salicylic acid in response to intentional TMV infection indicated conversion of salicylic acid to glucosyl - salicylic acid conjugate, as surmised from the absence of salicylic acid in the infection site of susceptible and tolerant varieties.

29. The practical utility of this biochemical marker in breeding programme was validated by estimating SA levels in TMV inoculated leaves of first & second filial and back cross progenies derived from the cross FCV Special x Va 770. Higher level of SA on TMV inoculation was detected only in resistant progenies. Plants with high and low SA levels followed the same segregation ratio as that obtained from visual necrotic symptoms. This proves the usefulness of salicylic acid as a supportive biochemical marker to facilitate selections in breeding programme.

30. Parallel increase of ribonuclease with viral multiplication in TMV susceptible variety supported the hypothesis of need for "host helper function" for the *de novo* synthesis of virus.

31. The absence of sudden spurt in ribonuclease enzyme in response to TMV infection in tolerant variety was demonstrated for the first time. This finding provided an experimental proof for the slower multiplication of TMV due to non-availability of helper function and resultant reduced symptom development in TMV tolerant varieties.

32. The mechanism of TMV tolerance in FCH 6248 was explained as due to absence of "helper function" in the host. This is a rare example of specific host plant mechanism to contain the disease damage. The process of degradation of host RNA has been minimized due to reduction in ribonuclease activity during infection. Absence of host genome helper function by lack of ribonuclease activity results in non-availability of raw materials for the *de novo* synthesis of virus in infected cells.

33. Measurement of ribonuclease enzyme activity in tobacco seedlings has been proved to be a useful, quick and reliable biochemical marker to identify tolerance to TMV infection.

34. Ribonuclease as a marker enzyme has fulfilled the need to have a rapid tool to identify tolerant variety at the early stage of plant growth (by 20 days). The traditional method takes about 120 days thereby increasing the selection efficiency by 6 folds.

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