Total phenol profile in some rice varieties in relation to infestation by Asian rice gall midge *Orseolia oryzae* (Wood-Mason)

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Estimation of total phenols at the stem bases in genetically related rice varieties in relation to the gall midge infestation revealed the role of phenols in expression of plant resistance involving hypersensitive reaction against the insect conferred by the gene *Gm2*. However, their role in other genetically diverse rice varieties with gall midge resistance was not clear.

THE Asian rice gall midge (RGM), *Orseolia oryzae* (Wood-Mason) (Diptera: Cecidomyiidae), is a serious pest of rice in several rice-growing countries of Asia, including India causing an estimated annual yield loss of over Rs 300 crores[1,2]. Upon hatching from eggs, laid on rice plants, maggots crawl down the plant between leaf sheaths to reach the apical meristem on which they feed. Maggot feeding causes formation of a tubular sheath gall called silver shoot. Further differentiation of the affected tiller is arrested and the tiller is rendered sterile. Pupa wriggles up the elongated gall and drills an exit hole that allows emergence of the adult fly. The main approach for the management of this pest has been through the development of resistant varieties. Though the mechanisms of resistance in rice against RGM are not fully understood, the level of resistance is generally complete immunity. No distinct oviposition preference is exhibited among resistant and susceptible rice accessions[3]. Early work suggested morphological features of rice plant contributing to the poor establishment of maggots in resistant cultivars[4], while later studies noted that maggots were present in equal numbers in both resistant and susceptible varieties[5]. A predominant antibiosis component leading to mortality of 1st instar maggots has been observed by many workers[6,7]. Some of the RGM-resistant rice genotypes express, subsequent to the pest attack, hypersensitive reaction (HR) which involves tissue necrosis at the site of insect feeding and leads to maggot mortality[8]. Other resistant varieties do not express HR but maggot mortality is noted as in the former group. These two groups are referred to as HR+ and HR– types, respectively.

Higher concentration of phenols in shoot apices of gall midge-resistant rice varieties Shakti, Leuang 152 (ref. 9), Ptb 18 (ref. 10), IET 7008, IET 7009 and Siam 29 (ref. 11) have been reported without any regard to the pest infestation. However, analysis of either the basal stem portion or the whole plant sample of twenty-nine RGM-resistant and susceptible rice varieties for total phenol content did not reveal any correlation with resistance[5]. Reddy[12] noted an increase in the total phenol content following RGM infestation both in resistant and susceptible rice varieites. Thus a clear association has not been shown so far to indicate that phenols play a role in RGM resistance. Since genetically diverse resistance mechanisms against RGM exist in rice varieties and some of these are induced by the pest attack, the role of phenols in RGM resistance, if any, needs to be studied under known genetic background and with reference to the pest attack. Here we report the total phenol profile in genetically heterogeneous and homogeneous plant materials in relation to RGM infestation and establish a clear role of phenols in conferring RGM resistance in HR + genotypes.

All the plant materials were obtained from the collection of RGM-resistant rice germplasm maintained at the Directorate of Rice Research, Hyderabad. Rice varieties, selected on the basis of the reaction, genetics and nature of resistance[13], like Phalguna with *Gm2* gene (HR+ type) resistant to RGM biotypes 1, 2 and 5; W 1263 with *Gm1* gene (HR– type) resistant to biotypes 1, 3 and 5; Suraksha deriving resistance from *Ptb* 18 (gene undefined) (HR+ type) resistant to biotypes 1, 2, 3 and 4 and TN1 lacking any resistance gene, formed the genetically heterogeneous set. Another set of rice varieties, based on the RFLP data pertaining to the 47 recombinant inbred (RI) lines in F5 or F6 generation from the cross Phalguna/ARC 6650 (RP 1579) generated with 48 single copy DNA probes distributed over all the 12 chromosomes[14], with three resistant and four susceptible RI lines were identified which displayed 33–97% genetic homogeneity (Table 1). Plants were grown in plastic trays as rows of 20–25 seedlings and exposed to gall midge biotype 1 adults (50 females + 10 males) 8–10 days after sowing. The next day the trays were transferred to a humidity chamber (RH > 90%) for egg incubation. Eggs hatched on the third day of oviposition and this was treated as 0 day of infestation. Stem bases, 2–3 cm in length, were cut from 5 seedlings per replication, pooled and fresh weight recorded before phenol extraction in methanol at 60°C for 20 min. Five replications per variety were maintained. Plants were sampled on 0, 1, 3 and 5 days after infestation. Total phenol content was estimated following Price and Butler[15] and expressed as mg g^{-1} fresh tissue. Data were subjected to analysis of variance (ANOVA) and the mean differentiated by LSD at 5% using IRRISTAT 4.0 software.

Results of the first set of genetically heterogeneous plant material (Figure 1) did not indicate a clear trend

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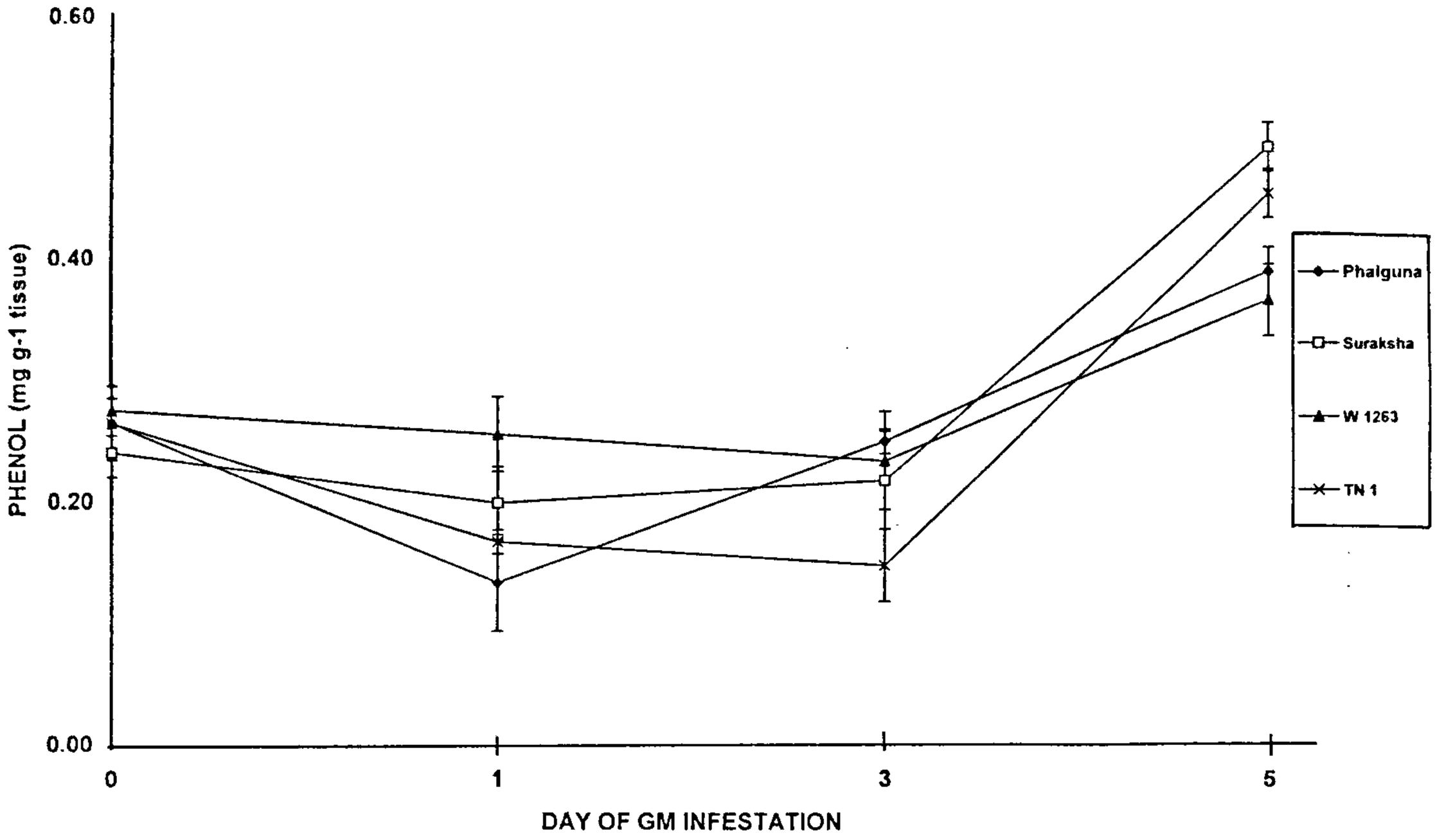

Figure 1. Total phenol profile at stem bases of genetically heterogeneous rice varieties in relation to rice gall midge infestation.

Table 1. Genetic homogeneity among the recombinant inbred lines of the cross Phalguna/ARC 6650 (RP 1579)

RI line no.	Per cent homogeneity*		
	RI19 (R)	RI38 (R)	RI43 (R)
RI24 (S)	81	81	79
RI28 (S)	33	33	33
RI30 (S)	35	35	35
RI45 (S)	97	95	97

*Based on polymorphism data obtained with 48 RFLP probes[14].
R, resistant; S, susceptible.

among the varieties. The day of infestation had a significant effect on the total phenol content ($F = 63.5$, $P < 0.001$; $df = 3$) but effect of varieties was not significant ($F = 1.29$, $P > 0.05$; $df = 3$). Such an increase in phenol content could not be related to gall midge resistance since the susceptible control TN1 also showed this trend as noted earlier[12]. Phalguna variety registered significantly higher levels of phenol as compared to TN1 only on day 3 after infestation. In the second set, there was a clear trend of increase in phenol levels in all the three resistant RI lines, viz. RI19, RI38 and RI43. There was a significant day of infestation × variety interaction ($F = 5.42$, $P < 0.001$, $df = 21$). All the resistant lines recorded significantly higher levels of total phenols when compared to the susceptible lines, viz. RI24, RI28, RI30 and RI45 (Figure 2) on day 5 after infestation. Even 3 days after infestation RI19 and RI38 displayed increased levels of phenol compared with the susceptible lines. The tissue necrosis as part of HR is noted in Phalguna, a HR+ type donor, between day 3 and day 5 after infestation by gall midge biotype 1 (ref. 8). Thus increase in phenol levels between day 3 and 5 after RGM infestation in resistant RI lines and no such corresponding increase in the susceptible RI lines which share 95–97% genetic homogeneity with the former group can be conclusively related to RGM resistance mechanism conferred by *Gm2* gene.

A wide range of allelochemic compounds present in plants play an important defensive role against insects and other herbivores. Phenolics have been associated extensively with the chemical defense of plants against microbes, insects and other herbivores[16]. These compounds have the ability to form insoluble complexes with proteins, act as enzyme inhibitors or are oxidized to toxic quinones. Several associations have been reported between phenolics and the resistance of plants to insect damage[17]. Expression of HR, common in plant–pathogen interaction, involves phenyl–proponoid pathway[18]. Expression of HR as part of plant resistance against insects is rare but not uncommon[19]. Thus rapid

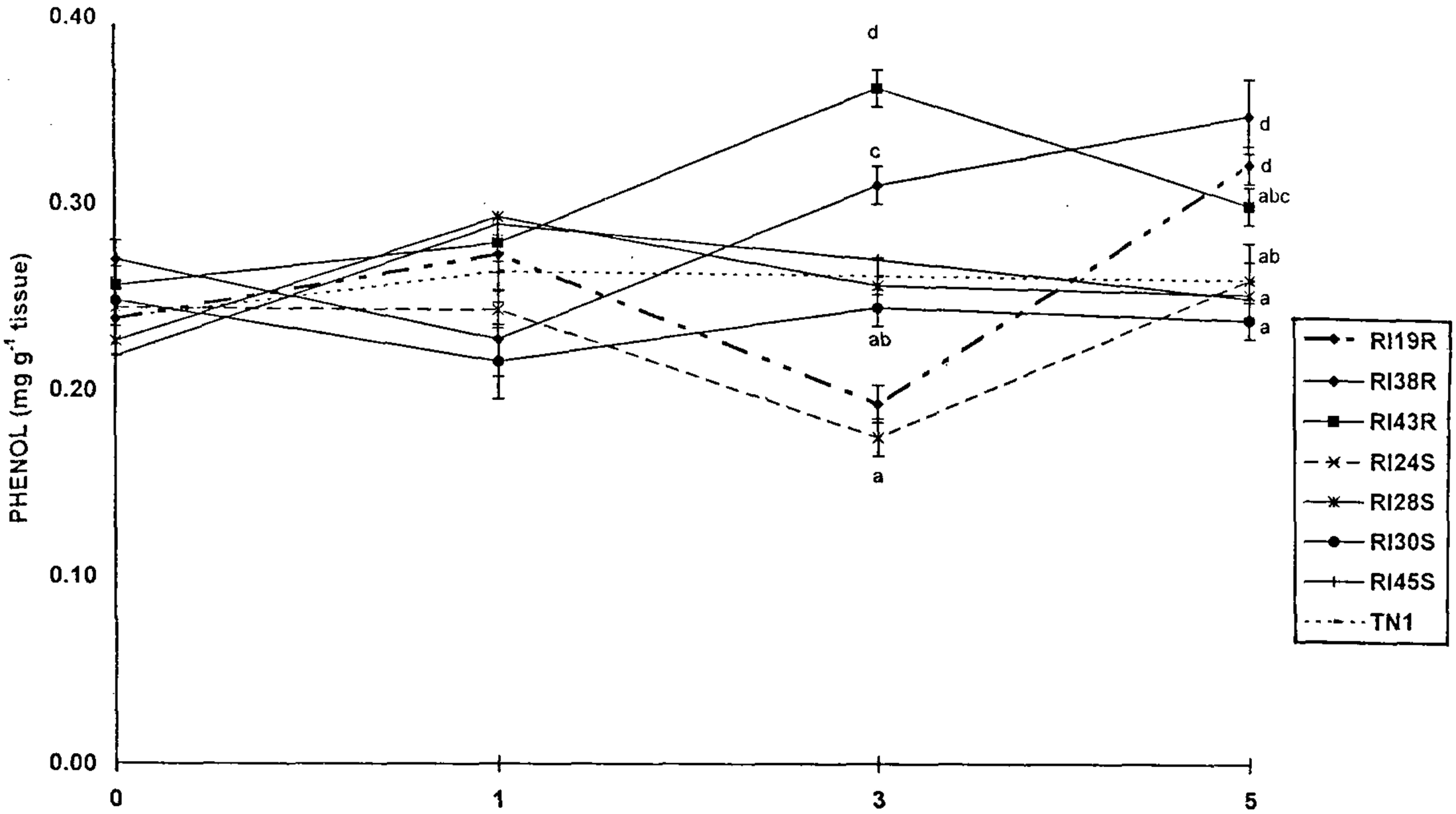

Figure 2. Total phenol profile at stem bases of genetically homogeneous rice varieties in relation to rice gall midge infestation.

accumulation of phenols in resistant RI lines following RGM infestation highlights the inducible biochemical pathway of expression of HR probably involving synthesis of phenolic precursors and their further oxidation into toxic quinones. It may be noted that once induced, the toxins produced are effective even against a virulent RGM biotype[8]. However, such a mechanism is not involved in the rice variety W 1263 used in the first set. A constitutive expression of a toxin of a different nature may be operative here against RGM. Such a diversity of expression of resistance against RGM among rice germplasm may have been the primary source of ambiguity in earlier reports. Slight increase in phenol content even in susceptible varieties may be associated with gall initiation process[20]. Phenols act as IAA oxidase inhibitors resulting in hyper-auxinity in gall tissue that leads to formation of nutritive tissue on which the gall formers feed. Thus both compatible and incompatible interactions in rice gall midge appear to follow an initial common biochemical pathway as has been highlighted in plant–microbe interactions[21].

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Received 31 December 1998; accepted 4 March 1999

Distribution of membrane-bound calcium and activated calmodulin in cultured protoplasts of sunflower (*Helianthus annuus* L.)

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Cultured protoplasts, isolated from the hypocotyl segments of seedlings of *Helianthus annuus*, exhibit rapid changes in intracellular-bound calcium and calmodulin (CaM) activation, in response to auxin (IAA, 10^{-5} M) treatment. Activities of bound calcium and CaM have been localized photomicroscopically, using specific fluorochromes – chlortetracycline (CTC) and trifluoperazine (TFP), respectively. Bound calcium accumulation is followed by an increase in Ca^{2+}–CaM activity. Bound calcium initially shows preferential accumulation in the nucleus, within 2 min of incubation of protoplasts in IAA-containing medium. The fluorescence gradually increases along the plasmalemma. Ca^{2+}–CaM activity shows similar but later (within 10 min of incubation) distribution in the cultured protoplasts. In the multicelled bodies, however, Ca^{2+}–CaM activity appears to be preferentially localized in the meristematic region, whereas bound calcium shows more uniform pattern of distribution. The percentage of protoplast populations exhibiting the above-stated changes in the distribution of bound calcium and calmodulin activation, varied between 70 and 85 in different experiments and their repititions. This indicates the important role of calcium and calmodulin activation in the manifestation of polarity.

SUNFLOWER has proved to be a relatively difficult plant for protoplast culture. A number of genotypes have been investigated[1]. Particular attention has been paid to the first stage of protoplast culture, so that the steps for further development can be optimized. Divisions in cultured protoplasts, their oriented growth and subsequent differentiation are believed to be under the control of ionic fluxes[2]. Intracellular calcium is involved in a large number of physiological processes and many external stimuli result in changes in intracellular concentration and compartmentalization of calcium ions and calmodulin[3,4]. There is increasing evidence that Ca^{2+} participates in the initiation and maintenance of polarity in plant cells[4] . In the light of these observations, we have undertaken a study of the distribution of bound calcium during the initial stages of protoplast culture in sunflower, together with an analysis of the distribution of activated calmodulin (Ca^{2+}–CaM complex) because of its dominant role in the regulation of calcium metabolism and cell division.

Monitoring intracellular free Ca^{2+} poses many problems in plant cells and the success of loading the specific fluorochrome depends on the plant in question and also the fluorochrome being used. Chlortetracycline (CTC), which has been used in the present work to localize intracellular calcium, has a good cell permeability and easily loads into plant cells[5,6] but it localizes membrane-bound calcium. Activated calmodulin (Ca^{2+}–CaM) can be detected by the use of a group of CaM inhibitors (phenothiazines, such as trifluoperazine; TFP)) which bind specifically with activated calmodulin forming a Ca^{2+}–CaM–phenothiazine complex[7,8]. TFP has been used in the present work to study the distribution of activated calmodulin in the cultured protoplasts.

Hypocotyls from 7-day-old *in vitro*, dark-grown seedlings were used for aseptic, enzymatic isolation of protoplasts. Hypocotyl segments (1 gm fw) were sliced and incubated in 5 ml of enzyme solution in plastic steriplates for 16 h in dark at 30 ± 2°C. The mixture was shaken gently for 10 min at the end of incubation and filtered through 80 µm stainless steel mesh. The protoplasts thus released were pelleted by centrifugation at 80 g for 5 min and washed thrice in the isolation medium (IM). This procedure results in a protoplast population free from cell wall debris. The composition of IM and enzyme mixture are as follows: IM (gm l^{-1}): NaCl 18; KCl 0.4; $CaCl_2 \cdot 2H_2O$, 6.13; MES 0.7 (pH adjusted to 5.6).

Enzyme mixture: Macerozyme R10 0.2%; cellulase TC 0.1%; pectinase boerozyme 0.5%. The individual enzymes (Serva Fine Chemicals Co, Germany) were dissolved in IM. Glassware used for protoplast isolation were sterilized by autoclaving at 15 lbs psi for 15 min. The enzyme mixture was filter-sterilized using sterilized filter assembly (pore size: 0.22 µm).

The viability of isolated protoplasts was tested after 5 min of incubation in 0.01% fluorescein diacetate

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