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Biochemical Analysis for Rice Germplasm Lines for Combat against Yellow Stem Borer, *Scirpophaga incertulas*: Implication for Varietal Selection of Rice in India

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Authors' contributions

This work was carried out in collaboration among all authors. Authors DSR and CC had designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors SKR and SNU managed the analyses of the study and performed statistical analysis. Author TM managed the literature searches and suggestions in writing first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Seven germplasm lines (IC No. 381538, 450535, 463380, 464140, 464186, 574807 and 578388) were categorised as best entries with resistance/ moderately resistant reaction at both vegetative (dead hearts) and reproductive (white ears) stages of the rice crop through field screening trials for two successive seasons *i.e.*, *kharif*, 2016 and *kharif*, 2017. Complex factors like behavioural, metabolic processes of the insects and biochemical constituents of the host plants are involved in

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resistance to rice stem borers. Exploiting the mechanism of induced resistance in rice through abiotic elicitors is the need of the hour. Keeping in view the damage of yellow stem borer and its influence on rice yield the promising seven entries were selected for biochemical analysis. The analysis revealed that entries with low sugars and low free amino acids and high total phenols, proteins, proline and silica content may confer resistance to rice yellow stem borer and can be subjugated in the breeding programme to develop resistant varieties.

Keywords: Rice; germplasm; yellow stemborer; bio chemical analysis.

1. INTRODUCTION

Rice is the main staple crop for more than half of the world's population. The major menace in rice cultivation among biotic stresses includes damage by insect pests. Among 320 insect species attacking the rice crop, the stem borers are reported to cause severe economic yield loss. The stem borer larvae infests the central shoot of the rice crop and their subsequent inter nodal penetration results in characteristic symptom of dead hearts during vegetative stage and white ears during reproductive stage [1] .The assessed yield losses of rice in India due to borers ranged from 30 to 80 per cent [2]. An infestation level of two per cent dead heart was declared as the economic threshold level for stem borer incidence to Basmati rice in Punjab [3]. Similarly, Asghar et al. [4] suggested an action threshold of five per cent dead hearts for effective and economic suppression of stem borer in rice ecosystem of Pakistan. Yield Loss Estimation Trial (YLET) revealed that for every ten per cent increase in white ears damage due to stem borer resulted in 1.02 g reduction in grain yield [5]. The per cent reduction in rice grain yield was predicted as 23.7, 41.7, 74.1 and 88.5 per cent for 10, 20, 50 and more than 80 per cent white ears [6].

Integrated pest management strategies entail the concept of host plant resistance as it is economical, compatible with control tactics, environmentally feasible and more appreciably farmers virtually do not need any skill in application techniques. Host plant resistance to yellow stem borer is ambiguous [7] because the varieties exhibiting resistance at dead heart stage were found susceptible at white ear stage indicating that the resistance at both the stages are independent and apparently none of the varieties developed so far have more than a moderate degree of resistance scale. However, the source from moderate resistance genotypes with genes conferring resistance was being tested, verified and analyzed for development of several other donors with high level of resistance. Field screening for 215 entries of rice germplasm was made at Agricultural Research station, Garikapadu, Krishna district, Andhra Pradesh, India by adapting augmented block design for successive seasons *i.e*., *kharif*, 2016 and *kharif*, 2017 to determine the relative susceptibility or resistance against the rice yellow stem borer, *S. incertulas*.

Among 215 entries screened, only seven were proven best imparting resistance or moderate resistance against stem borer infesting rice in terms of both dead hearts and white ears damage. Host plant resistance against rice yellow stem borer have indicated that in most cases the resistance was of biochemical nature. The phytochemicals involved in host plant acts as feeding deterrents, growth inhibitors, toxicants, ovipositional deterrents and repellents [8]. Hence, exploiting the mechanism of induced resistance in rice through abiotic elicitors is the need of the hour. Biochemical basis of resistance and general association with anatomical characters of the rice plant and their influence on conferring resistance was studies for the promising entries.

2. MATERIALS AND METHODS

Biochemical constituents, *viz.,* total sugars, total phenols, crude proteins, total free amino acids, proline and silica content were estimated from the samples of shoot apices collected from one month old rice plants of selected promising germplasm, susceptible check and local check (Table 1).

2.1 Extraction of Plant Samples

Uninfected vegetative shoot apices of 0.5 cm from 30 days old plants of test genotypes were collected after stripling leaves and leaf sheaths. The collected plant samples (five plants per genotype) were thoroughly washed with distilled water and dried under shade. One gram of plant

Table 1. The promising germplasm lines against yellow stem borer, *S. incertulas* infesting rice

S. No	IC no.	Dead Hearts (DH)						White Ears (WE)					
		kharif 2016		*kharif* 2017		Mean		*kharif* 2016		*kharif* 2017		Mean	
		% DH	Reaction	% DH	Reaction	% DH	Reaction	% WE	Reaction	% WE	Reaction	% WE	Reaction
1	381538	11.6	MR	14.3	MR	13	MR	10.8	MS	10.1	MR	10	MR
2	450535	16.9	MR	11.4	MR	14	MR	6.5	MR	3.5	R	5	R
3	463380	10.5	MR	10.8	MR	11	MR	4.3	R	2.9	R	4	R
4	464140	16.9	MR	13.4	MR	15	MR	5.6	MR	5.0	R	5	R
5	464186	16.8	MR	13.4	MR	15	MR	4.3	R	4.0	R	4	R
6	574807	16.3	MR	13.2	MR	15	MR	6.1	MR	4.1	R	5	R
7	578388	11.9	MR	10.8	MR	11	MR	4.0	R	3.2	R	4	R
Checks	TN1	42.2	S	35.1	S	39	S	22.7	S	17.6	S	20	S
	BPT5201	32.8	S	26.9	MS	30	MS	17.0	S	14.6	MS	16	S

MR: Moderately Resistant; R: Resistant; S: Susceptible

sample of all the genotypes were taken in separate conical flask, and 15 ml of 80 per cent ethanol was added. It was refluxed for 30 min on hot water bath. After boiling, the extract was cooled, and the pieces of tissues were ground thoroughly in a mortar with pestle in slight ethanol. The supernatant was decanted into another flask and residue was again re-extracted with small quantity of hot ethanol and decanted. The extract was filtered through Whatman no. 1 filter paper and made up to a known volume with 80% ethanol. The ethanol part of (alcoholic) extract was stored in refrigerator at 4°C and was used for the estimation of biochemical constituents.

2.2 Estimation of Sugars

The estimation of total and reducing sugars was done by following the method described by Somogyi [9]. For estimating the total sugars, hydrolysis of non reducing sugars to reducing sugars was done by adding one ml of 1 N hydrochloric acid to one ml of plant sample extract and was heated on a boiling water bath at 50ºC for 20 min. Later, it was cooled and a drop of phenolphthalein indicator solution was added. Then, 1 N sodium hydroxide was added drop wise until the solution turned pink because of excess alkali. The excess alkali was reneutralized with 0.1 N hydrochloric acid, which was added drop wise until the solution turned colourless and was made up to a known volume. One milliliter of hydrolysate for total sugars and one ml of plant extract for reducing sugars were taken separately in boiling tubes to which one ml of freshly prepared alkaline copper tartrate reagent was added and boiled in a water bath for exactly 20 min. After cooling under running tap water, one ml of arseno-molybdate reagent was added with immediate mixing. The volume was made up to ten ml with distilled water, and the blue colour developed was read after ten min at 510 nm and 620 nm for total and reducing sugars, respectively. Suitable blanks were prepared that were used to adjust the light transmission to 100%. A standard curve was prepared with different concentrations of standard glucose, which was used to calculate the unknown.

2.3 Estimation of Total Phenols

The estimation of total phenols in the plant tissue was determined following Folin-Ciocalteau method [10]. One milliliter of Folin-Ciocalteau reagent was added to one ml of the alcohol extract of the plant sample in a test tube followed by 2 ml of 20% sodium carbonate solution, and the mixture was heated on a boiling water bath for exactly one min. It was later cooled and made up to 20 ml with distilled water. The blue colour developed was measured in a spectrophotometer at 650 nm. The standard curve was prepared using different concentrations of standard catechol and the amount of phenols present in different samples of rice genotypes was estimated using the standard curve.

2.4 Estimation of Total Free Amino Acids

Total free amino acids were estimated by Ninhydrin method [11]. Alcohol free extract of plant samples (0.1 ml) was pipette out into separate test tubes, and one ml of ninhydrin reagent was added and mixed well. The volume of each test tube was made up to 2 ml by using distilled water. All the test tubes were heated in a boiling water bath for 20 min. Then, 5 ml of diluents solution was added while the test tubes were still on the water bath and mixed well. Meanwhile, a blank was prepared by taking 0.1 ml of 80% ethanol and one ml of ninhydrin reagent was added, mixed, and was made up to 2 ml. After 15 min of boiling, tubes were cooled under running tap water and absorbance of purple colour was measured against reagent blank at 570 nm by using spectrophotometer. The standard curve was prepared using different concentrations of standard leucine, and the amount of total free amino acids in different samples of rice genotypes was estimated using the standard curve.

2.5 Estimation of Crude Protein

The micro-Kjeldahl method was followed for estimation of crude proteins. Half a gram of finely ground dry plant sample was placed in a boiling tube to which a pinch of digestion mixture and 15 ml of concentrated sulfuric acid was added. These tubes were kept for digestion in Kjel-Plus (KPS-012L) provided with vacuum pump for one hour. This was later allowed to cool and transferred to distillation apparatus. A 100 ml conical flask containing 25 ml of 4% boric acid with a few drops of mixed indicator (2 parts of methyl red + 1 part of methylene blue) was placed under the condenser, the tip of which was dipped in boric acid solution. Required amount of sodium hydroxide solution was added to the sample. The distillate was titrated against 0.1 N HCl until the original pink-red colour

restored. This method gives an estimate of total nitrogen content in samples, which was later converted to crude proteins by using conversion factor (*i.e*., %N 5.95).

2.6 Estimation of Proline

Estimation of Proline by Acid-Ninhydrin method [12] was followed. One gram of plant samples were homogenized in 10 ml of 3% aqueous sulfo-salicyclic acid. The homogenate was filtered through Whatman no. 2 filter paper. Two ml of glacial acetic acid and 2 ml of acid ninhydrin were added to the test tube containing 2 ml of plant extract. The residue was boiled on water bath for one h by placing the test tube in ice bath. Four ml of toluene was added to the reaction mixture and stirred well for 30 to 50 sec. The toluene layer was separated and warmed to room temperature. The intensity of red colour developed was then measured at 520 nm by using spectrophotometer. A series of standards with pure proline was run in a similar way to prepare standard curve. The amount of proline in the samples was estimated using the standard curve prepared from pure proline (range 0.1-36 *µ*mole) and express as fresh weight basis of sample.

> Protein/g tissue = [(*µ*g proline/ml x ml toluene) / 115.5(molecular weight of proline) X 5 / g sample]

2.7 Estimation of Silica

The per cent of silica as per the method described by Hesse [13] was followed. Five grams of oven dried and powdered leaf sample was transferred to a flat shaped silica basin which was weighed previously. The contents were heated over a hot plate to oxidize organic matter and later the basin was transferred to muffle furnace maintained at 300ºC. Allowed the ashing to proceed slowly and when no more glowing carbon seen, gradually raised the temperature of muffle furnace to a very dull red heat (500-550ºC). The ash was moistened later with little water and basin was covered. Added 40 ml of dilute HCl (1:1) and the basin covered with clock glass was placed on a water bath and digested for 20-30 min. Later the contents were removed and rinsed the cover, added one ml of conc. HNO_3 to oxidize any ferrous salts and to evaporate the contents to dryness. The heating process was continued for half to one hour to dehydrate silica. The contents were later moistened with 10 ml of dilute HCl (1:1) and 50 ml of water was added and warmed on the bath tub. Later filtered the contents through Whatman No.44 filter paper and collected the filtrate in a suitable volumetric flask. Later the insoluble residue from the basin was transferred to the filter using a rubber tipped stirring rod to remove silica particles adhered to the sides of basin and washed with hot dilute HCl (1:20). Later, cooled and weighed the insoluble residue (contains essentially of silica and small amounts of other elements). The loss in weight then corresponds to the silica content.

The data pertaining to various bio chemical constituents in selected rice accessions were tabulated and analysed through CRD analysis of one way ANOVA by using suitable transformations. The critical difference values were calculated at p=0.05 and mean values were compared using Duncan's Multiple Range Test (DMRT).

2.8 Correlation between Bio Chemical Constituents and Resistance in Rice Germplasm

The yellow stem borer damage in terms of both per cent dead hearts (% DH) and per cent white ears (% WE) were correlated with biochemical constituents of the promising rice germplasm and check varieties.

3. RESULTS AND DISCUSSION

The biochemical factors *viz*., total sugars, total phenols, free amino acids, protein, proline and per cent silica content may influence the resistance mechanism in rice varieties against yellow stem borer. Hence, the promising (selected) seven rice germplasm lines were evaluated for biochemical bases of resistance and compared with check varieties (Table 2 and Fig. 1).

3.1 Total Sugar Content

In the selected genotypes the total sugar content (mg g-1) ranged from 13.38 (C-1372) to 20.65 (C-497) as against highest recorded in local check (TN1) and susceptible check (BPT 5204) with 31.18 and 32.30 mg g--1, respectively. The total sugar contents in chronological descending order among seven promising entries were C-497 (20.65) > C-903 (19.41) > C-685 (18.25) >C-1247 (15.66) ≥ C-858(15.66) > C-901(15.65) > C-1372 (13.38). The bio chemical analysis in

relation to sugars witnessed that higher the sugar content comparatively higher was the pest incidence. The results were in accordance with the findings of CRRI [14], Bharathi [15] and Loka Reddy et al. [16] who strongly confirmed that the soluble sugars were high in susceptible varieties than resistant ones. Maheswari et al. [17] verified the phloem sap samples of resistant and susceptible rice genotypes and admitted that total sugar content was highest in C20 R and TN1 varieties which were highly susceptible to rice pests.

The reports of Vijaya et al. [18] and Vanitha et al. [19] supplemented the present findings who also disclosed that soluble sugar contents were more in susceptible varieties compared to resistant varieties. These findings were in disagreement with the reports of Peraiah and Roy [20] who found high sugar content in resistant rice genotypes (RR 270-56, JGL 11650, NDR 3110 and NDR 2063) than susceptible checks. Studies by Sai [21] stated that the total sugars or phenols in healthy samples of resistant and susceptible varieties are not indicative of any role relating to resistance against insect pests. The results by [22] depicted that the amount of total sugars, reducing sugar and non-reducing sugars in all susceptible genotypes was found higher compared to resistant and moderately resistant accessions.

3.2 Total Phenols

Total phenols (mg/100 g) were higher in C-858 (7.63) followed by C-1247 (7.09), C-903 (6.98), C-901 (6.54), C-497 (6.11), C-685 (5.95) and C-1372 (5.34). Relatively lowest phenol contents were realized in TN1 and BPT 5204 with 4.89 and 4.96 mg/100g, respectively. The results were in accordance with Das [23] who reported that rice variety with high phenol content exhibited resistance against stem borer, leafhoppers and planthoppers and declared phenols as feeding deterrents. In contrast, Sai [21] and Vijaya et al. [18] stated that the total phenols had no role relating to resistance in rice pests. But Sogawa [24], Pathak and Khush [25] confirmed that resistant rice genotypes contain more phenolic compounds than susceptible varieties. Chandramani [26] affirmed that the high phenol containing rice plants (MDU 5) amended with organics exhibited lowest yellow stem borer infestation.

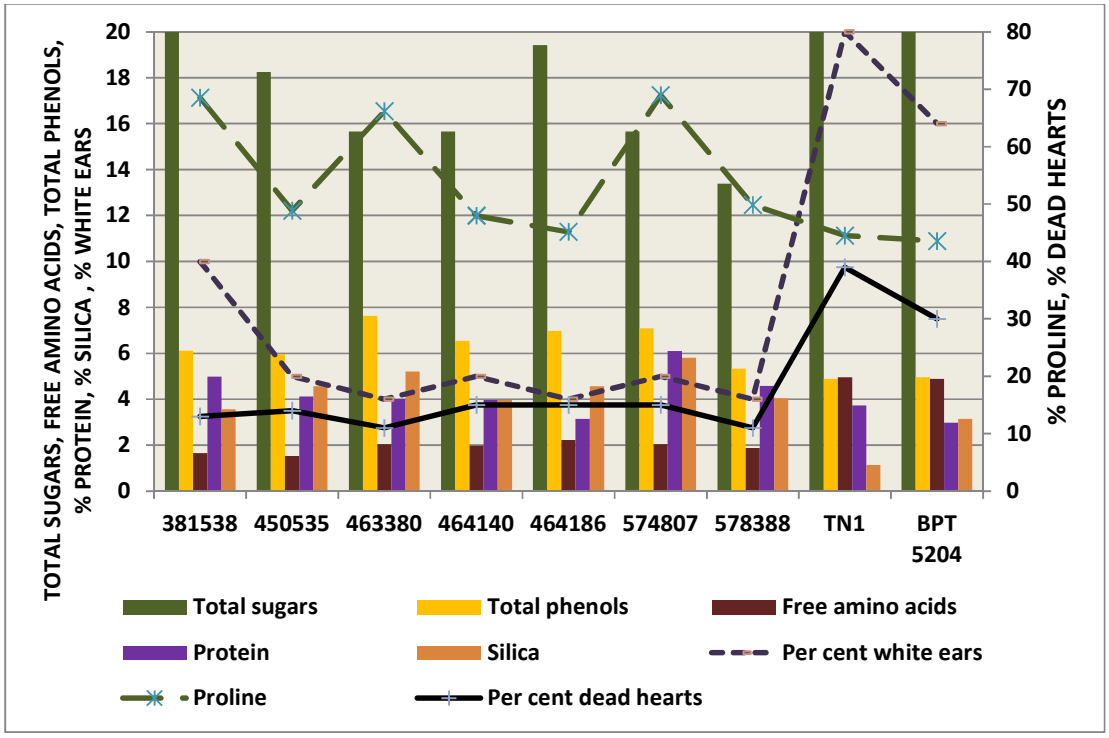

Fig. 1. The biochemical analysis for the promising rice germplasm lines against yellow stem borer, *S. incertulas*

Table 2. The biochemical analysis for the promising rice germplasm lines against yellow stem borer, *S. incertulas*

S. no.	Entry no.	IC No.	Total sugars (mg g^{-1})	Total phenols (mg/100g)	Free amino acids (mg/100g)	Protein (mg g^{-1})	Proline (ppm)	Silica (%)
1	C-497	381538	20.65^{c}	6.11^{cd}	1.65^{a}	4.98	68.53^{d}	3.57^{a}
2	C-685	450535	18.25^{bc}	5.95^{bc}	1.54^{a}	4.12	48.85^{c}	4.57^{b}
3	C-858	463380	15.66^{ab}	7.63^{f}	2.05^{a}	4.01	66.18^{d}	5.21^{c}
4	C-901	464140	15.65^{ab}	6.54^{cde}	1.98^{a}	3.98	47.98^{bc}	3.98^{a}
5	C-903	464186	19.41^{c}	6.98^{d}	2.23^{a}	3.15	45.15^{b}	4.57^{b}
6	C-1247	574807	15.66^{ab}	7.09^{ef}	2.04^{a}	6.1	68.98^{d}	5.81^{c}
7	C-1372	578388	13.38^{a}	5.34^{ab}	1.87^{a}	4.58	49.85^{c}	4.04^{ab}
Checks		TN1	31.18^{d}	4.89^{a}	4.96^{b}	3.73	44.51^{ab}	3.20^{a}
		BPT 5204	32.30^{d}	4.96^{a}	4.89^{b}	2.98	43.55^{a}	3.15^{a}
CD (P=0.05)			2.85	0.61	0.82	NS	3.81	1.14
CV			5.17	4.75	3.98	5.48	3.19	5.78

Maheswari et al. [17] noticed high total phenol content (22.52 ppm) in leaf sheaths of the rice resistant genotype (IR 54742-22-19-3R). Elanchezhyan et al. [27] suggested that higher concentration of total phenols in the resistance rice genotypes acts as contributing factor for tolerance with antibiotic effect against stem borer. Reports by [22] also inferred that higher level of total phenols and ortho-dihydroxy phenol were found in resistant and moderately resistant rice accessions as against susceptible accessions, indicating that these defensive compounds contributing towards the rice yellow stem borer resistance. Studies by [28] also stated that the phenol content was maximum in the resistant rice entries ranging from 6.26 to 7.72 mg/g and minimum in the highly susceptible entries ranging from 2.53 to 3.21 mg/g.

3.3 Free Amino Acids

The free amino acid content in the promising genotypes in chronological descending order were 2.23 (C-903) > 2.05 (C-858) > C-1247 (2.04) > C-901 (1.98) > C-1372 (1.87) > C-497 (1.65) > C-685 (1.54). In check varieties the free amino acids content was comparatively higher in local check (BPT 5204) and susceptible check (TN1) with 4.89 and 4.96 mg/100g, respectively. The results revealed that low concentrations of free amino acids impart resistance to stem borer in rice. Sogawa and Pathak [29] 1970, Sogawa [23] and Bharathi [15] also stated that aromatic amino acids in low concentrations exhibit an inhibitory effect on stem borer infestation.

Vidhyachandra et al. [30] stated that the stems of both resistant (TKM6) and moderately resistant genotypes (Ratna) expressed less amino acids content than susceptible genotype (IR8). Koyama [31] strongly affirmed that amino acids content enhance the nymphal growth of hoppers. In contrast Vijaya et al. [18] indicated that free amino acids content was more in resistant varieties than susceptible ones. However, Vanitha et al. [19] and Kumar et al. [32] emphasized that free amino acids were lower in resistant varieties compared to susceptible varieties like TN1.

3.4 Proteins

With respect to protein content in rice genotypes analyzed in the present study there was no significant difference between the promising genotypes, susceptible check and local check varieties and the protein content (mg g-1) varied from 2.98 to 4.98. The results were in close conformity with Sai [21] and Vijaya et al. [18] who said that there was no role of protein as biochemical content in host plants conferring resistance or susceptibility against rice pests. In contrast, Peraiah and Roy [20] elucidated that high crude protein content was realized in resistant rice genotypes (RR 270-56, JGL 11650, NDR 3110 and NDR 2063) than susceptible checks.

Padhi and Chatterji [33] also supported the above reports by declaring that protein content in the leaf sheath of rice varieties was higher in expressing resistant against yellow stem borer than susceptible ones. Sujatha et al. [34] confirmed that the protein, nitrogen, zinc and manganese contents in rice plants were negatively correlated with resistance. But the biochemical analysis by Shahjahan [35] expressed that the resistant cultivars (BR1, DA26 and Kalizira) contained lower percentage of moisture, protein and fat than the susceptible cultivars (BR14, BR2 and Pajam).

3.5 Proline

The proline content among the selected rice germplasm ranged from 43.55 ppm (BPT 5204) to 68.98 ppm (C-1372). The proline contents estimated in promising germplasm lines in descending order were 68.53, 66.18, 49.85, 48.85, 47.98, 45.15, 44.51 and 43.55 ppm in C-1372, C-903, C-858, C-1247, C-901, C-685 and C-497, respectively. The lowest proline content was registered in check varieties with 44.51 and 43.55 ppm in TN1 and BPT 5204, respectively. The proline accumulated in plant tumours (infested) functions as a competitive antagonist of gamma-amino butyric acid (GABA) dependent plant defense, interfering with the GABA induced degradation of quorum-sensing signal.

The results of biochemical analysis stated that higher proline content in resistant cultivars of rice imparts resistance to yellow stem borer attack and were in concurrence with the findings of Rajadurai and Kumar [36] who revealed the presence of higher amount of total phenols and proline in rice varieties as the factors imparting resistance to the rice pests. Roy et al. [37] noticed the accumulation of higher concentration of proline in BPH infested rice plants. Contrast reports by Vijaya et al. [18] stated that the biochemical profiles in relation to rice gallmidge infestation involves decreased proline and indole acetic acid content in the growing apical meristem of resistant genotypes compared to susceptible genotypes.

Table 3. The correlation matrix between damage by *S. incertulas* and biochemical constituents of rice germplasm

S. no.	Biochemical components	Per cent DH	Per cent WE
1	Total sugars	0.951	0.942
2	Total phenols	-0.690	-0.730
3	Free amino acids	0.973	0.900
4	Protein content	-0.458	-0.385
5	Proline content	-0.527	-0.339
6	Silica content	-0.647	-0.741

3.6 Silica

The silica content was lowest in susceptible check variety (TN1) and local check variety (BPT 5204) with 3.20 and 3.15 per cent, respectively. Maximum silica (%) content was observed in the promising genotype C-1247 (5.81%) followed by C-858 (5.21%). The next germplasm lines in descending order of silica content were C-685 (4.57%) ≥ C-901 (4.57) > C-1372 (4.04) > C-901 (3.98) > C-497(3.57).

From the analysis, it was inferred that silica content conferred resistance to yellow stem borer infesting rice and the same was confirmed by Sasamoto [38] who stated that there was an increase in the silicon content of rice plants, when raised in silicon supplied soils and parallel decrease in the susceptibility to the stem borer, *C. suppressalis*.

Djamin and Pathak [39] also revealed a highly significant negative correlation (r = -0.617) between silica content of stem and the per cent dead hearts by Asiatic rice borer, *C. suppressalis* in the rice varieties. Panda et al. [40] reported that yellow stem borer larvae meagrely attacks resistant rice plants due to the presence of high silica and crude fiber content in their stems. Sujatha et al. [32] Bandong and Listinger [41] and Hosseini et al. [42] also expressed that silica contents in rice were positively correlated with host plant resistance against yellow stem borer. Chavan et al. [43] stated that higher silica content in moderately resistant rice varieties exhibited significantly negative correlation with per cent dead hearts (r = -0.756) and per cent white ears (r = -0.896) due to yellow stem borer infestation.

Rajamani et al. [44] also viewed that the dead hearts due to yellow stem borer were negatively correlated with plant silica content in rice.

3.7 The Correlation Matrix between Biochemical Components and Damage by *S. incertulas*

It was evident from the correlation studies that, total sugars and free amino acids exerted non significant positive correlation with per cent dead hearts (0.951 & 0.973) and per cent white ears (0.942 & 0.900) damage by yellow stem borer. The total phenol, protein, proline and silica contents exhibited non significant negative correlation with yellow stem borer damage in terms of both dead hearts (-0.690, -0.934, -0.527 & -0.647) and white ears (-0.730, -0.864, -0.339 & -0.741), respectively (Table 3).

4. CONCLUSIONS

In this context, it can be viewed that the biochemical components of the rice plant plays a crucial role in relation to resistance or susceptibility to the biotic stresses like stem borer attack. The identical consideration was also stated by Rajadurai and Kumar [34] who suggested that the rice entries with high silica, phenol, proline contents and low chlorophyll, total sugars, reducing sugars, total soluble protein contents can be exploited in the breeding programme to develop resistant varieties against the rice yellow stem borer, *S. incertulas*.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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