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Differential morphological, cytological and biochemical responses of two rice cultivars to coumarin

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ABSTRACT

We investigated the differential responses of two contrasting rice cultivars (susceptible BS-2000 and less susceptible BR-41) to coumarin at the cytological, biochemical and morphological levels. Both rice cultivars, showed similar trend in the relative sensitivities of specific root parameters to coumarin: total number of root hairs >total number of lateral roots > radical length. However, the light and electron microscopic observations of seedling root and leaf cells exhibited differential responses to coumarin exposure. BR-41 showed higher ribosomal density (RD), more mitochondria in root cells, larger starch granules in leaves and more vacuoles (smaller size) than BS-2000. Coumarin also induced different antioxidant responses in two cultivars. BR-41 showed 23% higher superoxide dismutase (SOD), 16% higher catalase (CAT) and 17% higher peroxide dismutase (POD) activities than BS-2000 when exposed to 400 µM coumarin. However, malondialdehyde (MDA) contents and electrolyte leakage were significantly higher in BS-2000, which exhibited 8 and 10 % higher MDA levels and 49 and 45% higher electrolyte leakage than BR-41, when exposed to 200 and 400 µM coumarin, respectively. Our results suggested that biochemical and cytological responses and root morphological characteristics jointly affect the rice resistance to coumarin stress.

Key words: Allelochemicals, allelopathy, coumarin, cytological and biochemical, enzymes, genotype, morphological, *Oryza sativa* L., response.

INTRODUCTION

Allelopathy refers to any direct or indirect harmful or beneficial effect of one plant on another through the release of organic chemicals (allelochemicals) into the environment (16,33). In plants, these chemicals can be continuously released by donors into their immediate environment as water leachates, root exudates in soil, volatiles in the air, or as decomposition of plant residues (24). All plant structures, including roots, stems, leaves, flowers, fruits, and seeds, are capable of releasing chemicals into the environment (45). Plant responses to allelochemicals are often species-specific and concentration dependent, being often stimulatory at low concentrations and inhibitory at higher concentrations (7). Understanding the mechanisms underlying plant responses to

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allelochemicals released into the soil is vital to determining the role of allelopathy in plant communities.

Coumarins (1,2-benzopyrones) are a group of natural products derived from the general phenylpropanoid pathway (10). Benzopyrone compounds also include many other allelochemicals (furanocoumarins and pyranocoumarins), which are widely distributed in both natural and agricultural plant communities (11). Scopoletin (7-hydroxy-5-methoxycoumarin), umbelliferone (7-hydroxycoumarin) and esculetin (6,7-dihydroxycoumarin) represent the most studied coumarins associated with plant-plant allelopathic interactions (17,18). Coumarin (2H-chromen-2-one) has been identified in numerous plant species and accumulates to particularly high levels in tonka bean (*Dipteryx odorata*), vanilla grass (*Anthoxanthum odoratum*), woodruff (*Galium odoratum*), mullein (*Verbascum* spp.), bison grass (*Hierochloe odorata*), and sweet grass (*Hierochloe odorata*) (46). Due in part to its ubiquitous presence in plant cells, large quantities of coumarin are released into the environment, suggesting that this compound exerts a significant influence on ecological interactions and plant community structures (4,33).

In treated plants, coumarin inhibits the root growth (2,6,39), mitochondrial respiration and photosynthesis (32), nutrient mobilization (1) and nitrogen metabolism (3). Studies on the effects of coumarin exposure in plants have largely focussed on primary roots of various plant species (5,6,23). Relatively little informationis available about its comparative effects on root growth of different genotypes within the same species.

Rice (*Oryza sativa* L.) possesses considerable genetic variation for abiotic tolerance within its cultivated gene pool (31). In addition, differential transcriptional responses to salinity stress have been documented in both shoots and roots of contrasting rice genotypes (13). This study aimed to investigate the differential responses of two contrasting rice cultivars to coumarin at the cytological, biochemical and morphological levels.

MATERIALS AND METHODS

Rice seeds and chemical materials

Coumarin was purchased from Sigma (St. Louis, MO, USA). The solutions were prepared by dissolving requisite amounts of coumarin in 0.1% ethanol and control consisted of 0.1% ethanol. Seeds of rice cultivars BR-41 and IR-64 were obtained from the International Rice Research Institute (IRRI; Los Baños, Philippines), the Minghui and Huanjingxian cultivars were provided by Prof. Guiquan Zhang, South China Agricultural University, and cultivar BS-2000 was obtained from the Rice Research Institute, Kala Shah Kaku, Pakistan. Rice seeds were surface-sterilized with 1% NaOCl for 30 min, rinsed with distilled water, and pre-germinated in Petri dishes for 3 d prior to use in bioassays.

Screening of rice cultivars and allelochemicals

Uniformly grown seedlings of rice were transferred to Petri dishes containing filter paper dipped in 20 ml of 200 μM coumarin. Petri dishes were maintained in completely randomized design with three replicates, which were sealed with parafilm and incubated in a growth chamber at 24-26°C with 150 $\mu Mm^{-2}sec^{-1}$ light and 12/12 light/dark

photoperiod. The root length, shoot height and number of lateral roots (>1 cm) were recorded after 7 d.

Root morphology

To assess the root morphological adaptations, rice cultivars BR-41 and IR-64 were grown in soil-filled pots containing 0, 100, 200 and 400 µM concentrations of coumarin (added at 20 ml per pot after every 2-days) for 7 d. To assess the developmental response of the BR-41 and IR-64 cultivars to continuous stress, radical length, number of lateral roots, and number of root hairs were recorded.

The root structures of both rice cultivars at each coumarin concentration were examined after harvesting shoots and carefully removing root systems from soil. Whole root systems were spread in a plastic transparent tray filled with 3 mm deep water to minimize adhesion and overlap of individual branches. Scanned images of root systems were developed using an STD 1600 scanner (Reagent Instrument Inc., Quebec, Canada). The numbers of lateral roots on each radical were quantified using the scanned images. Branching density was calculated by the number of lateral roots divided by the total radical length (cm). Slides were also prepared for microscopic evaluation of root hair densities within 1 cm zones along the root axis, which were determined by counting root hairs from the tip, middle and origin. The total number of root hairs per root system were calculated by the average number of root hairs × root length × number of lateral roots.

Light microscopy

The seedlings of rice cultivars BR-41 and IR-64 were grown in petri dishes in continuous contact with 20 ml coumarin for 3 d. Root specimens for light microscopy were excised using glass knives mounted on a Sorvall Porter-blum MT2-B ultramicrotome (Ivan Sorvall Inc., Norwalk, Conn., USA). Sections were picked up with a wire loop and floated onto droplets of 10% acetone on a glass slide. The slide was dried on a warming tray at 50˚C. For staining, the slide was placed on a hot plate at 100˚C, flooded with staining solution for 2 min, rinsed with distilled water, and dried on a warming tray at 50˚C. The staining solution contained 1% toluidine blue and 1% azure II in 1% borax. Specimens were observed under bright field. Microscopic evaluations were conducted to monitor the location of cells viewed under transmission electron microscopy and to compare cell sizes and other histological changes between treated and untreated cells in roots of both rice cultivars.

Transmission electron microscopy

Root tips (approximately 3 mm length) and first leaves of rice seedlings of rice cultivars BR-41 and IR-64 germinated in coumarin (0 and 200 µM) solution for 3 d were rinsed in distilled water thrice and fixed for 2 h in a modified Karnovsky's fixative (20) containing 2% glutaraldehyde and 2% paraformaldehyde in cacodylate buffer (pH 7.0). Root tips were post-fixed for 1 h in 1% OsO_4, then stained *en bloc* overnight in 0.5% aqueous uranyl acetate at 4˚C. Specimens were first dehydrated by a graded series of 30-100% ethanol for approximately 20 min at each step, then transferred to absolute cycloparaffin for 20 min. Specimens were then infiltrated in a 3:1 mixture of absolute cycloparaffin and resin for 1 h at room temperature, transferred to 1:1 mixture of absolute cycloparaffin and resin for 4 h, and finally Spurr's resin overnight. Thereafter, specimens

were embedded in capsules containing embedding medium and heated at 70˚C for 9 h in Spurr's low viscosity epoxy resin (36). Thin sections (approximately 80 nm) were cut with a diamond knife mounted on an ultramicrotome (LKB 2088 Ultrotome V), and picked up with etched copper grids. Copper grids were washed by successively dipping them into 0.1 M HCl, distilled water and 97% EtOH. Copper grids containing thin sections were stained with 2% uranyl acetate followed by lead citrate for 15 min (30). Specimens were observed under transmission electron microscope (TEM).

Plasma membrane permeability assays

Loss of membrane integrity (an indicator of cellular damage) was determined in terms of ion leakage from roots of rice cultivars BR-41 and IR-64 by measuring the electric conductivity of bathing media containing different concentrations of coumarin (100, 200, 400 µM). Roots from 3-plants per treatment were collected after a 7-days exposure to coumarin solution and cut into 1 cm segments. Samples were washed thrice with deionized water to remove surface-adhered electrolytes. Roots were then placed in stoppered vials containing 10 ml of deionized water and incubated at 25˚C on a rotary shaker platform at 100 rpm. The electrical conductivity of bathing solutions (Lt) were determined using a model DDS-11A conductivity meter (Leici Instrument Co., Shanghai, China) after 24 h. Samples were then heated to 120˚C for 20 min, and a final conductivity reading (Lo) was obtained upon equilibration at 25˚C. The electrolyte leakage was defined as Lt/Lo and expressed as percent.

Enzyme assays

Germinants of rice cultivars BR-41 and IR-64 were grown for 3 d in petri dishes containing different coumarin treatment solutions as described above. Enzyme extracts were prepared from flash-frozen root tissues ground using a mortar and pestle in liquid nitrogen. Approximately 0.1 g aliquots of pulverized material were then homogenizedin 1.5 ml of 50 mM sodium phosphate buffer (pH 7.8), then centrifuged at 15000×*g* at 4˚C for 10 min. The supernatant was collected, stored at 4˚C, and used for the analysis of superoxide dismutase (SOD), catalase (CAT) and peroxide dismutase (POD). SOD activity was measured using the method described by Beauchamp and Fridovich (8). POD activity was determined using the method of Malik and Singh (25).CAT activity was assayed using the method described by Camark and Marschner (12). All assays were performed in triplicate and all experiments were repeated twice.

Malondialdehyde content analysis

Lipid peroxidation was measured in terms of malondialdehyde (MDA) content (14). Root samples of rice cultivars BR-41 and IR-64 were extracted with trichloroacetic acid (TCA, 0.1% w/v) and centrifuged at 10000 x *g* for 10 min. After centrifugation, 1 ml of supernatant was added to 4 ml of 0.5% thiobarbituric acid (TBA, in 20% TCA). The mixture was incubated at 95^{o}C for 30 min and then quickly cooled over ice and centrifuged at 10000×*g* for 10 min. The absorbance of the supernatant was determined at 532 nm and corrected for non-specific absorbance at 600 nm. MDA content was calculated using an extinction coefficient of absorbance of 155 mM^{-1} cm^{-1} and expressed as nmol g^{-1}FW. All measurements were made in triplicate.

Statistical analysis

For all experimental treatments described, three replicates were maintained in a completely randomized design. Sigma Plot 12.0 (Systat Software, Inc.) package for windows was used for statistical analysis. The data for screening of rice cultivars were analyzed with one-way analysis of variance (ANOVA), with the significant differences among means identified by Fisher's LSD test ($P<0.05$). Data for root morphology and enzymatic analysis of rice cultivars BR-41 and IR-64 exposed to different coumarin concentrations were analyzed with two-way ANOVA, with significant differences among means identified by Fisher's LSD test($P<0.05$). Before using ANOVA, the data were tested for normality and homogeneity of variance.

RESULTS

Screening of rice cultivars

To examine whether significant variation in tolerance to coumarin exposure could be detected in rice, various accessions were first screened to compare the inhibitory effects of coumarin on root and shoot lengths, and on the number of lateral roots produced (Fig 1). Overall, the inhibitory effects of coumarin (200 µM) observed on shoot growth for all tested cultivars were significant. Shoot growth of BS-2000, BR-41, Minghui, IR-64 and Huanjingxian were reduced by 45.5, 19.8, 27.6, 32.5 and 31.6%, respectively. The shoot length of BS-2000 was inhibited 25% more by coumarin as compared to BR-41. The inhibitory effects were more pronounced on root growth than shoot growth in all tested cultivars. Coumarin at 200 µM inhibited the root growth of BS-2000, BR-41, Minghui, IR-64 and Huanjingxian by 76.5, 47.7, 62.2, 72.3 and 67.9%, respectively (Fig 1). The radical length of rice cultivar BS-2000 was 27% more inhibited thanBR-41(Fig 1). Among all 5-tested cultivars, BS-2000 proved sensitive to coumarin and therefore designated as 'susceptible', while BR-41 was least sensitive and termed 'less susceptible' (Fig 2). The numbers of lateral roots varied significantly between BS-2000 and BR-41.The less susceptibleBR-41cultivar exhibited high branching density than the susceptible BS-2000 cultivar. Coumarin at 200 µM reduced the numbers of lateral roots of all tested cultivars> 54.4% (Fig 1).

Root morphological responses

As mentioned above, BS-2000 and BR-41 exhibited the largest differences in coumarin susceptibility among the rice cultivars tested, and were therefore chosen for use in comparative follow-up studies. Root responses to coumarin were first investigated under natural soil conditions using the two cultivars. Three-day-old germinants of coumarin-susceptible BS-2000 and less susceptible BR-41 were exposed to soils containing 0, 100, 200, and 400 µM coumarin for 7 d. Overall, the numbers of lateral roots, branching density, and total number of root hairs were reduced relative to controls in both rice cultivars grown in the presence of 200 µM and 400 µM coumarin (Table 1). The mean number of root hairs per centimeter were similar in two cultivars at lower coumarin concentrations, but differed at 400 µM coumarin (approximately 57% more root hairs/cm observed for BR-41 - Table 1). The 200 µM coumarin application inhibited the radical

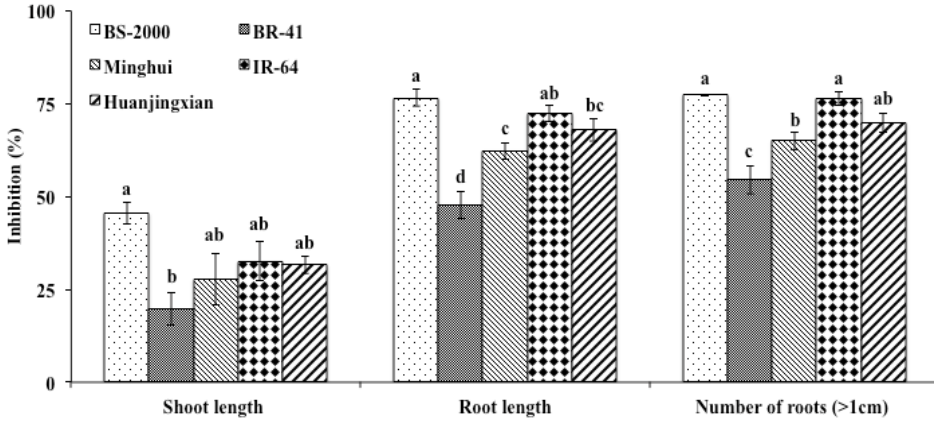


Figure 1. Effects of coumarin (200 µM) on seedling growth of 5- rice cultivars. Values represent the inhibition percentage relative to controls for different cultivars. Significant differences ($P<0.05$ using LSD Fisher's test) among cultivars indicated by different letters according to one-way ANOVA.

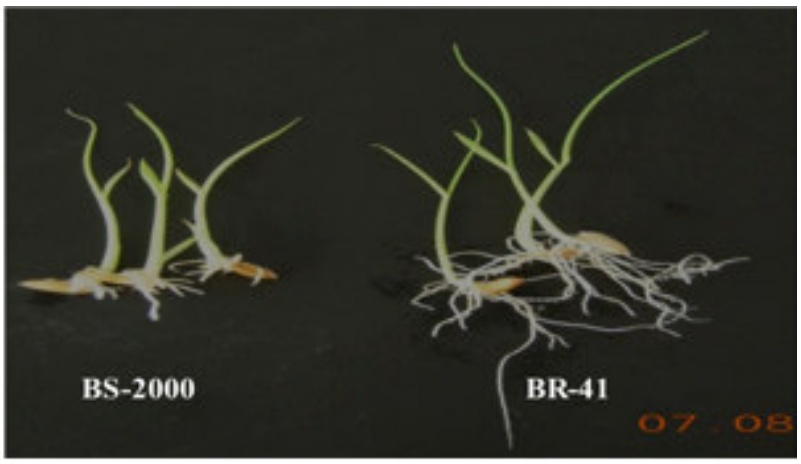


Figure 2. Rice seedlings of two cultivars (BS-2000 and BR-41) exposed to 200-µM coumarin

length, number of lateral roots, branching density and total number of root hairs in BS-2000 by 46, 58, 23 and 87 %, respectively, while those of BR-41 were inhibited by approximately 27, 52, 36 and 76 %, respectively relative to control (Table 1).

In both cultivars, a similar trend in the effects of coumarin on specific root parameters was observed (Table 1). For example, numbers of root hairs present were drastically inhibited parameter in both rice cultivars (87% inhibition for BS-2000 and 76% inhibition for BR-41), followed by lateral root formation (58% and 52%), while radical root lengths were less sensitive (46% and 27%). The order of sensitivity observed for both cultivars was total root hairs>lateral root>radical root>shoot (Fig. 1 and Table 1).

Table 1. Effects of coumarin concentration on root growth parameters of rice cultivars BS-2000 and BR-41

Rice Cultivar	Coumarin Conc (μM)	Radical length (cm)	Number of lateral roots	Branching density	Number of root hairs/cm	Total number of root hairs
BR-41	Control	4.4±0.4b	4.8±0.5a	1.10±0.07a	12.8±1.5a	267±20.6ab
	100	4.8±0.1b	5.2±0.13a	1.10±0.04a	11.8±0.88a	293±15.9a
	200	3.2±0.06c	2.3±0.10c	0.7±0.03b	8.1±0.53b	62±2.2d
	400	2.2±0.15d	1.3±0.16d	0.6±0.04bc	4.4±0.52c	12±0.8e
BS-2000	Control	6.0±0.29a	2.6±0.22c	0.4±0.06cd	12.8±0.41a	195±8.2c
	100	6.1±0.08a	3.3±0.16b	0.5±0.03c	13.2±0.30a	263±15.03b
	200	3.2±0.15c	1.1±0.07ed	0.31±0.02d	7.4±0.37b	24±2.93e
	400	1.4±0.11e	0.6±0.05e	0.5±0.12c	2.8±0.17d	2±0.49

Values are mean ± standard error (n=3). Significant differences ($P<0.05$ using LSD Fisher's test) among treatments in the same column are indicated by different letters according to two-way ANOVA. Branching density was calculated by the number of lateral roots divided by total radical length (cm).

Cytological responses

Light micrographs of both rice cultivars showed significant structural changes in response to coumarin exposure. Deterioration of root cortical cells was observed in both cultivars, but the extent of this deterioration was greater in BS-2000 (Fig. 3). Application of coumarin widened the cortical cells in both rice cultivars than control roots, shown by arrow. Cell walls of cortical cells were well defined in BR-41 as compared to BS-2000 after coumarin treatment. In addition to the inhibition of overall root system growth by coumarin, electron micrographs of root cells also showed obvious changes to subcellular structures of both rice cultivars (Figs. 4, 5). Cells of BS-2000 in coumarin-treated roots showed fewer amyloplasts containing a reduced number and smaller starch granules, invaginated plastids, irregularly shaped and lobed nuclei, and higher amount of lipids as compared to BR-41. BR-41 exhibited an increased number of vacuoles of various sizes. BS-2000 cells possessed a large central vacuole, while vacuolar density was reduced than in BR-41. Nuclei in BR-41 root cells were typically evenly spherical and contained prominent nucleoli after coumarin exposure (Fig. 4) but show some damage shown by arrow. Electron micrographs also indicated that rice cortical cells of untreated roots had well-developed organelles, such as the Golgi apparatus (dictyosomes), mitochondria, rough endoplasmic reticulum (RER), and abundant polyribosomes in both rice cultivars (Fig. 4A, C and E). Coumarin-treated cells of BS-2000 roots were more deteriorated thanBR-41 (Fig. 5A, 5B and 5C and 5D). This deterioration is also oblivious between comparisons of stressed BS-2000 cells and stressed BR-41 cells even when comparing BS-2000 at low magnifications (Fig 4B and 4D). A poorly-defined network of endoplasmic reticulum (RER) was observed in cells of both rice cultivars (BS-2000 being the least well-defined) after coumarin exposure (Fig. 4B).

Electron micrographs of BR-41 root tip cross sections showed that columella statocytes contained typical amyloplasts packed with starch granules, numerous mitochondria and darkly staining lipid granules in the cytoplasm (Fig. 5C and 5D).

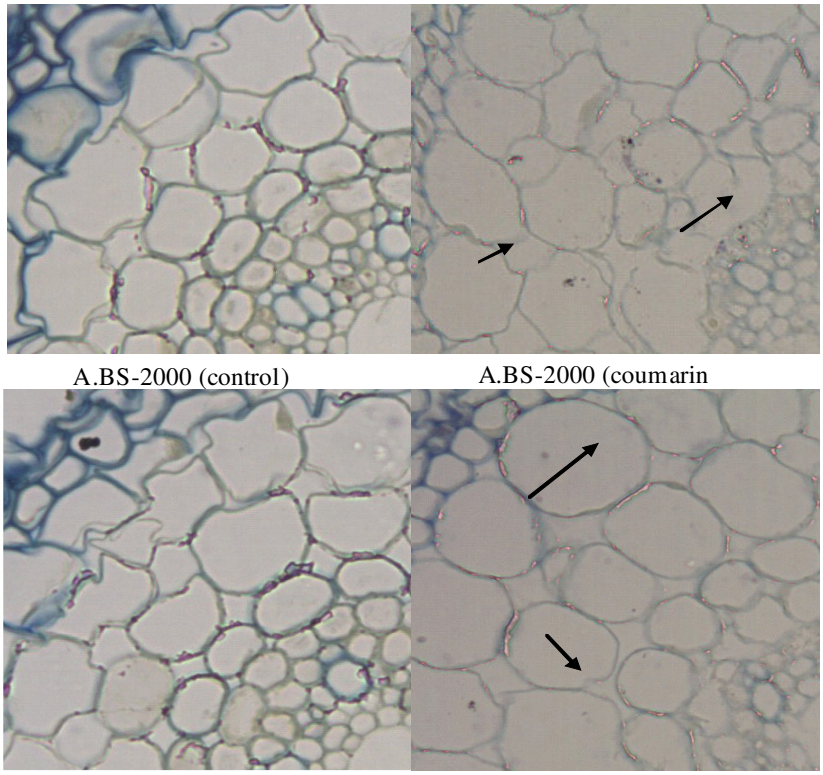


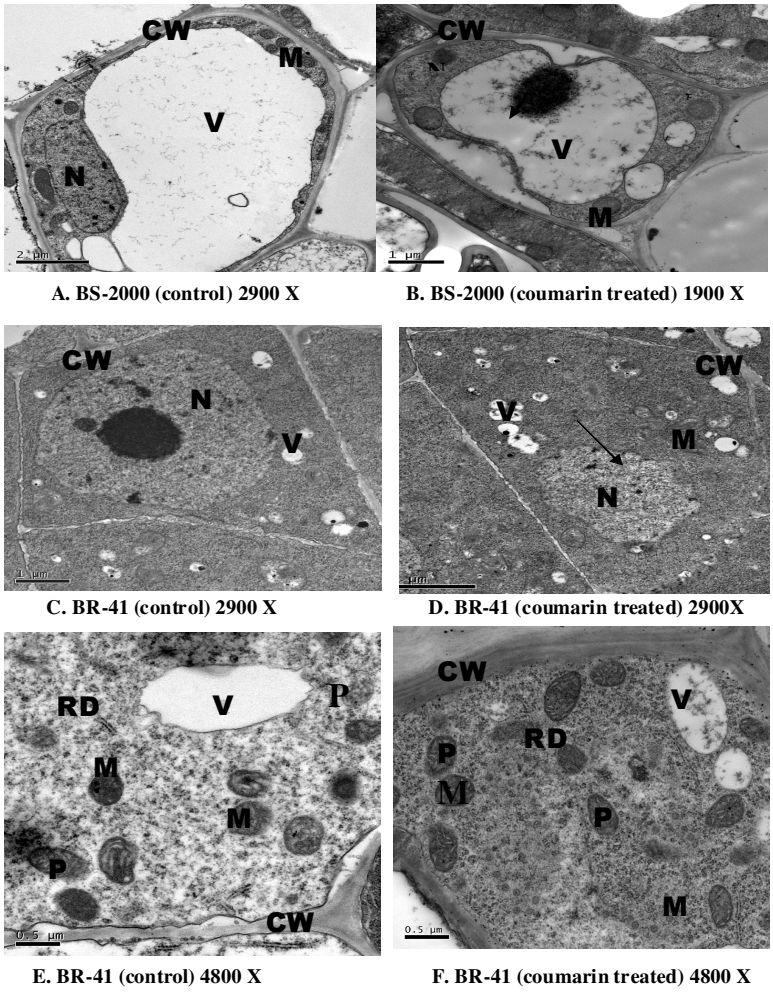
Figure 3. Light micrograph (approximately 0.1 mm^2 area shown) of root cross sections taken approximately 1 mm above the root tip of rice cultivarsBS-2000 (susceptible) and BR-41 (less susceptible) treated with coumarin (200 µM).

Electron micrographs of BR-4 leaf chloroplasts showed numerous starch granules and well-developed thylakoid structures than BS-2000 chloroplasts exposed to 200 µM coumarin (Fig. 5).

Biochemical responses

Potential loss of cellular membrane integrity due to coumarin exposure was examined by analyzing the electrolyte leakage from roots of cultivars BR-41 and IR-64 in bathing media containing different concentrations of coumarin (100, 200, 400 µM).The amount of electrolyte leakage did not vary significantly between the two cultivars following control treatments (Fig. 7A).Exposure to coumarin at 200 and 400 µM did, however, reveal significant differences between BS-2000 and BR-41. In BS-2000, electrolyte leakage was approximately 2-folds higher than BR-41 at both the 200 and 400 µM coumarin concentrations (Fig. 7A).

Levels of lipid peroxidation following exposure to coumarin were assessed by monitoring malondialdehyde (MDA) contents in roots of both cultivars. The BS-2000



A. BS-2000 (control) 2900 X **B. BS-2000 (coumarin treated) 1900 X**

C. BR-41 (control) 2900 X **D. BR-41 (coumarin treated) 2900X**

E. BR-41 (control) 4800 X **F. BR-41 (coumarin treated) 4800 X**

Figure 4.Electron micrograph of root tips of rice cultivarsBS-2000 (susceptible) and BR-41 (less susceptible)treated with coumarin (200 µM).CW, V, M, P, N, RD represent Cell Wall, Vacuoles, Mitochondria, Plastid, Nucleus and ribosomal density, respectively.

cultivar exhibited 7, 8 and 10% higher MDA levels than BR-41 at 100, 200 and 400 µM, respectively (Fig. 7B).

The activity of SOD in roots of both rice cultivars increased significantly in response to exposure to coumarin than controls (Fig. 8A), however the difference between the two cultivars was significant only at the highest coumarin concentration tested (400 µM). Similar trends were observed for POD and CAT activities. The POD activity in roots of both rice cultivars increased with increasing coumarin concentration, but BR-41 exhibited 8, 6, 10 and 17 % higher POD activities as compared to BS-2000 at 0, 100, 200

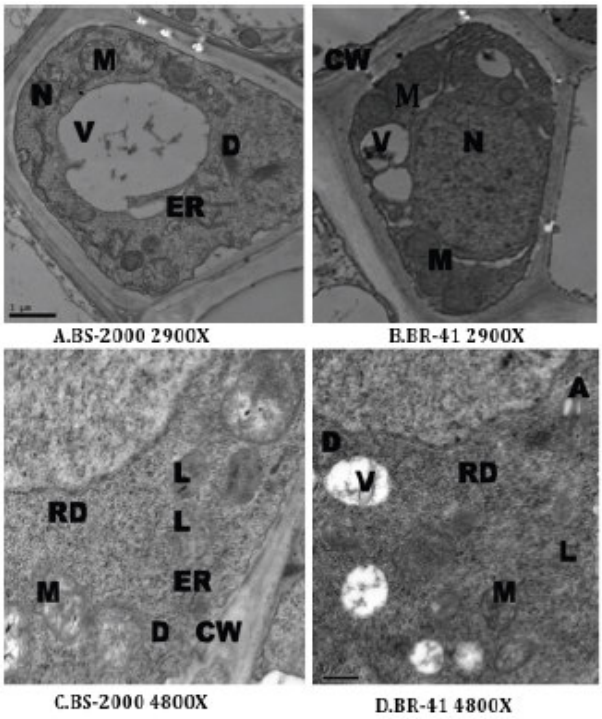


Figure 5. Electron micrographs of root tip cells of rice cultivarsBS-2000 (susceptible) and BR-41 (less susceptible) treated with coumarin (200 µM). L, ER, M, CW, D, A, RD, N and V represent Lipid Granules, Endoplasmic Reticulum, Mitochondria, Cell Wall, Dictyosomes, Amyloplasts, Ribosomal Density, Nucleus and Vacuoles, respectively.

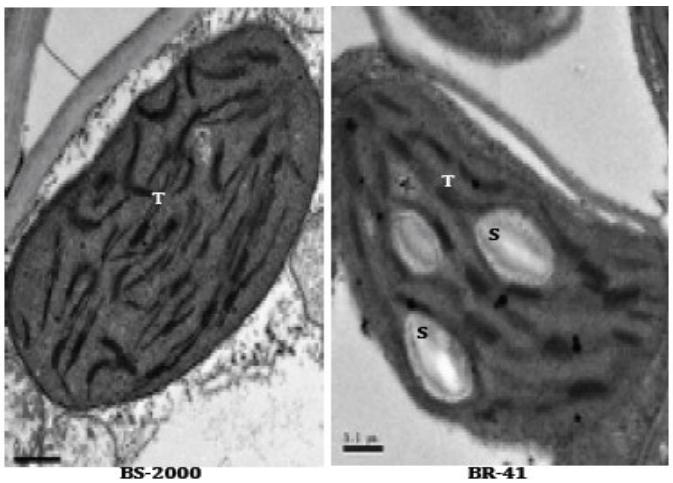


Figure 6. Electron micrographs (4800×magnification) of chloroplasts in leaves of rice cultivars BS-2000 (Susceptible) and BR-41 (less susceptible) treated with coumarin (200 µM). "S" represents Starch and ''T'' represents Thylakoid.

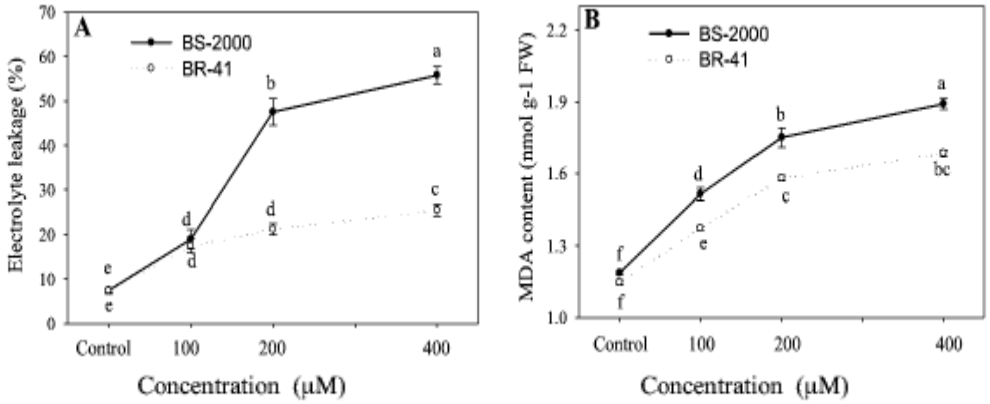


Figure 7. Electrolyte leakage (A) and malondialdehyde (MDA) content (B) from roots of rice cultivars BS-2000 (susceptible) and BR-41 (less susceptible) treated with different concentrations of coumarin. Values are mean ± standard error (n = 3). Significant differences ($P<0.05$ using LSD Fischer's test) among treatments are indicated by different letters above or below curves according to two-way ANOVA.

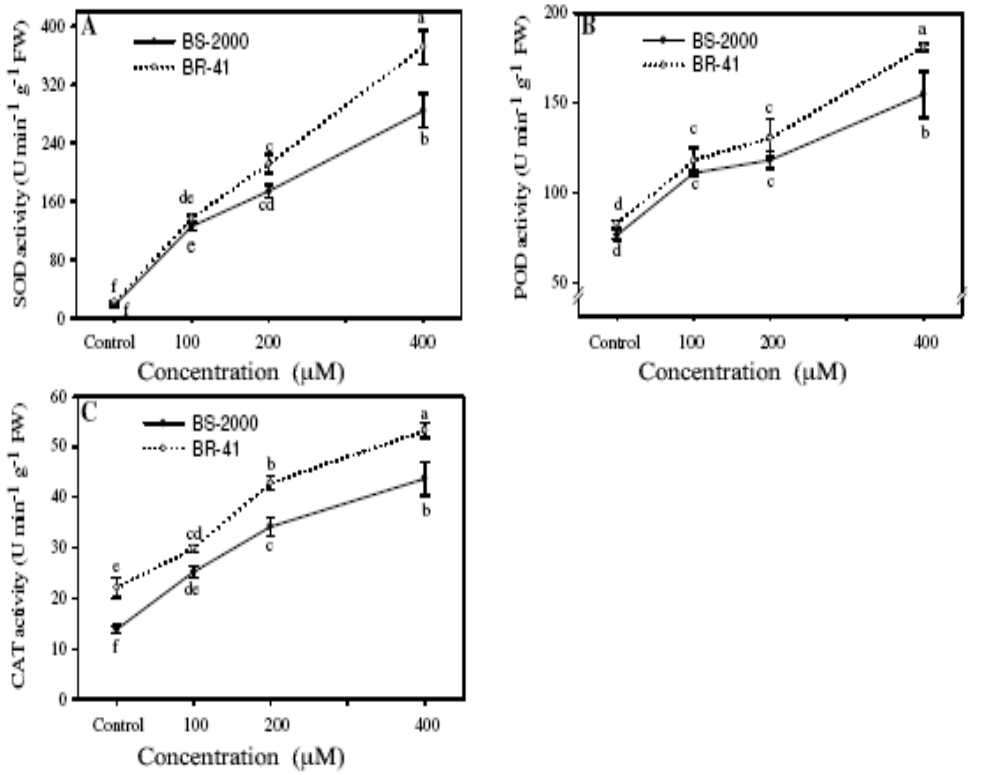


Figure 8. Effect of different concentrations of coumarin on the enzymatic activities of SOD (A), POD (B) and CAT (C) in roots of rice cultivars BS-2000 (susceptible) and BR-41 (less susceptible). Values are mean ± standard error (n=3). Significant differences ($P<0.05$ using LSD Fischer's test) among treatments are indicated by different letters above or below curves according to two-way ANOVA.

and 400 µM, respectively (Fig. 8B). The activity of CAT also increased steadily with increasing coumarin concentrations in roots of both rice cultivars, and as was observed for SOD and POD activities, significant differences between the two cultivars wereseenonlyin400 µM treatments. BR-41 showed 36, 13, 20 and 20% higher CAT activity than that of BS-2000 at 0 (control), 100, 200 and 400 µM coumarin, respectively (Fig. 8C).

DISCUSSION

Our results revealed that the five rice genotypes tested differed substantially in their responses following exposure to the common plant allelochemical coumarin (Fig. 1). Although coumarin reduced the radical length, number of lateral roots, and total numbers of root hairs of both rice cultivars, inhibition was more pronounced in BS-2000 (designated 'coumarin-susceptible') than BR-41 ('less susceptible') (Fig. 2). A similar trend was observed for both cultivars in terms of the parameters most strongly affected by coumarin: total number of root hairs >number of lateral roots > radical root length (Table 1). Given that allelochemicals are spatially concentrated within the uppermost soil layers (21) that are typically occupied by fibrous-rooted plants, coumarin could play an important ecological role. Abenavoli *et al.* (2) reported that tap-rooted plant species adapt better to allelochemical stress than fibrous-rooted species. Our results indicate, however, that BR-41 (fibrous-rooted, higher root branching density) is actually better able to cope with coumarin-imposed stress than BS-2000 (tap-rooted, lower root branching density). A possible reason for why the less susceptible BR-41 cultivar copes well with coumarin stress could be due to their differential physiological, biochemical and cytological characteristics. The higher branching density might helpful to cope well to coumarin stress as observed in Pb stress to rice cultivars. Yang *et al.* (41) showed that Pb-tolerant rice lines develop more adventitious roots following Pb treatment. Similarly, average radical root lengths of untreated plants of the susceptible rice cultivar were longer than those of the tolerant cultivar (Table 1), which could facilitate the uptake and absorption of coumarin from the surrounding soil (Table 1). Yu *et al.* (42) showed that the trans-cinnamate tolerant figleaf gourd (*Cucurbita ficifolia* Bouché) take up less trans-cinnamic acid than cucumber (*Cucumis sativus*), which is trans-cinnamate susceptible. Subsequently Zhang *et al.* (43) reported that the down-regulation of cell cycle-related genes in cucumber could have an impact on selective uptake mechanisms. It may be the same case in rice as well, given that a higher branching density was observed in the less susceptible rice cultivar (Table 1). The increased ability of the BR-41 cultivar to tolerate coumarin could also be attained through the release of intracellular Ca^{2+} (42) and certain organic acids (41). Senadheera *et al.* (34) reported that a salt-tolerant rice cultivar exhibited lower Na^+ influx, reduced Na^+ translocation to the shoot, and maintenance of a lower Na^+: K^+ ratio due to the differential expression of membrane transporters (34).

Not only the growth of whole root systems of both rice cultivars affected by coumarin exposure, but also damage to both rice cultivars was very evident at the subcellular level, as revealed by light and electron microscopy (Fig 3, 4, 5 and 6). The cytological responses observed in root cells were different in BR-41 and BS-2000, which could be inferred from the differences in the ultrastructural changes observed in the

presence of coumarin for the two cultivars (Figs. 4, 5 and 6). The structural changes observed within root tips demonstrated that the degree of cellular disorganization was dependent upon the sensitivity of the corresponding cultivar to the inhibitor. The major differences observed between the two rice cultivars were the shape and number of vacuoles, the number of mitochondria, and number of lipid globules present. BR-41 had smaller-sized but a larger number of vacuoles than BS-2000, a larger number of mitochondria, and also fewer lipid globules (Figs. 4 and 5). Lipid degradation is known to increase during germination to fulfill the energy requirements of growing seedlings (27). Energy stored in the form of lipids is released via the glyoxylate cycle of plant seedlings in association with mitochondria. The high amounts of lipid globules present in root cells of BS-2000 seedlings may suggest a deficiency in the utilization of energy reserves required for cellular growth and proliferation. A lack of lipid degradation has also been observed in root cells of *S. alba* in response to allelochemicals. An inhibition of lipid catabolism by coumarin may result in reduced ATP production, which could represent an additional cause for growth reduction in BS-2000. Inhibition in ATP production has been observed in mitochondria of *Cucumis sativus* hypocotyls under flavonoid stress (37).

Membrane disruption, as indicated by solute leakage, was higher in BS-2000 as compared to BR-41 (Fig 7A). An increase in membrane permeability can be due to peroxidation of membrane polyunsaturated fatty acids, resulting in the formation of several by-products, including MDA (26). It has been previously suggested that allelochemicals may in some cases be toxic to microorganisms due to their ability to disrupt membranes (19). MDA content was higher in BS-2000 as compared to BR-41 at all coumarin concentrations tested (Fig. 7B). MDA content is often used as an indicator of lipid peroxidation and membrane damage in plants (35). The observed MDA differences showed a differential response to coumarin in the susceptible (BS-2000) and less susceptible (BR-41) rice cultivars. The peroxidation of cellular membrane lipids severely impacts membrane functionality and integrity, and can result in irreversible damage to cells (38). According to our results membrane injury level was higher in BS-2000 as compared to BR-41, in the presence of coumarin.

Plants possess both enzymatic (SOD, POD, CAT, etc.) and non-enzymatic defense systems for the detoxification of various types of allelochemicals (22). In the present study, rice roots exhibited higher activities of SOD, POD and CAT under coumarin stress (Fig 8). Increased levels of SOD activity indicate the presence of oxidative stress caused by excessive generation of O^{-2}, presumably resulting from coumarin exposure. Importantly, BR-41 (less susceptible to coumarin) displayed higher activities of antioxidant enzymes as compared to BS-2000 (coumarin-susceptible). Increases in antioxidant enzyme activities have been observed in plant roots after exposure to allelochemicals in previous studies (44). Induction of antioxidant enzymes may play an important role in plant resistance to allelochemicals in the environment. This discrepancy in antioxidant enzyme activities and membrane permeability between the two rice cultivars may be due to differences in NADPH oxidase activity and the intrinsic capacity of two rice genotypes. An increase in superoxide radicals at higher concentrations of coumarin could suggest a loss of normal reduction of oxygen to water and other electron carriers during mitochondrial electron transport (9). In plants subjected to stress-imposing conditions, the productions of ROS are typically increased and their levels are controlled by antioxidant enzymes (28). In fact, recently Lupini *et*

al. (23) showed that coumarin induces ROS and plays vital role in gravitropic response. Our data regarding catalase activity showed that the less susceptible rice cultivar had higher CAT activity as compared with the sensitive rice cultivar (Fig. 8). Saline-tolerant tomato plants exhibited up-regulation of antioxidative enzymes upon exposure to high saline conditions (29), similar to our finding of higher SOD and POD activities in the less susceptible rice cultivar as compared to the sensitive cultivar (Fig. 8). In most biotic and abiotic stress conditions, an overproduction of ROS is responsible for oxidative damage (28) and inhibition of root viability (15). Catalase (CAT) was found to be a crucial factor for maintaining the redox balance during oxidative stress in tobacco plants (40).

Coumarin is a kind of allelochemical and many plant species contain this allelochemical. In summary, coumarin can cause inhibitory affects to rice root system and released coumarin into environment by various plants can play crucial role in plant-plant allelopathic interactions. This study demonstrated that coumarin exposure cause phytotoxic activity to rice due to membrane damage, an increase in antioxidant enzyme activities, and apparent loss of root system integrity of rice cultivars. On the other hand this study also put emphasis how two rice cultivars with differential morphology-ical characteristics overcome coumarin stress. The coumarin-susceptible rice cultivar BS-2000 showed greater growth inhibition, increased cellular damage, higher MDA content and electrolyte leakage, and lower activities of antioxidant enzymes (SOD, POD and CAT) as compared with the less susceptible BR-41 cultivar. Coumarin stress adversely impacted over all root system morphology, and also compromised numerous subcellular structures in root cells. Our results suggest that cytological and biochemical alterations, along with morphological changes, could jointly contribute to the enhanced tolerance of a rice genotype to environmentally derived coumarin. Further studies addressing coumarin uptake, coumarin exposure levels under field conditions, and plant detoxification pathways for coumarin will be necessary to fully elucidate the mechanisms involved in the ability of rice plants to adapt to the presence of this allelochemical.

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