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Water stress induced physiological and biochemical changes in *Piper betle* L. and *Ricinus communis* L. plants and their effects on *Spodoptera litura*

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ABSTRACT

Plant responds to stresses by developing adaptive changes, which some times are complex and can be synergistically or antagonistically adjusted by the plant defense mechanisms. We studied the abiotic stress induced changes in physiological, biochemical and oxidative level reactions caused by drought stress in *Piper betle* L. (Piperaceae) and *Ricinus communis* L. (Euphorbiaceae) plants and their influence on the feeding performance of herbivore, *Spodoptera litura*. Under drought stress, the leaf chlorophyll and relative water content (RWC) in both test plants were decreased than controls. In piper and castor plants, the quantitative analysis was carried out on leaf biochemicals (carbohydrates, proteins, amino acids, flavonoids and phenols) under drought conditions. The decrease in the individual phenolic acids in both plants due to stress caused by water deficit was determined using HPLC analysis. The reduced levels of primary metabolites were evident in both plants, while flavonoid content enhanced along with amino acid content in castor plants. These changes created a favorable environment for *S. litura* and increased the feeding rate in both drought affected castor and piper plants than their normal plants. Increased levels of antioxidative enzymes [superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT)] in leaves of *P. betle* and *R. communis* indicate their defensive and/or protective role against the ROS generated under drought conditions. It was concluded that drought conditions induced the defense responses in both *P. betle* and *R. communis* plants, which regulated the herbivore feeding performance.

Key words: Antioxidative enzymes, biochemicals, drought stress, oxidative stress, *Piper betle, Ricinus communis, Spodoptera litura*

INTRODUCTION

Plants are subjected to different types of stresses, during their growth and development. Stress (biotic and abiotic) on plants result in upregulation of genes associated with the production of metabolites such as proteinase inhibitors, toxins etc. Feeding stress by insects and mechanical damages in tomato and potato plants caused the accumulation of trypsin and chymotrypsin like protein inhibitors as a direct response (9, 17). Abiotic stresses (e.g., drought, extreme temperature, cold, heavy metals, or high salinity) impair plant growth and productivity worldwide (38). However, plant responses

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to abiotic stresses associated with the induction of reactive oxygen species are less studied compared to biotic stress induced reactive oxygen species (ROS) and these ROS metabolites can act as signalling molecules related to plant acclimation (10). Water stress induces the physiological and biochemical changes in pigeon pea plant (37). Abiotic stress directly impact the photosynthetic apparatus and is characterized by stomatal closure, turgor loss, reduction of relative water content (RWC) and in severe cases photosynthesis is curbed, metabolism disrupted and finally leads to plant death (3, 26). This severely impairs plant growth and development. Along with biochemical and physiological changes, the increase in drought conditions may enhance the frequency and severity of insect population outbreaks (42), because the environmental stress increases the plant's susceptibility to insect herbivory by altering leaf chemistry and whole plant physiology (29).

In many plants, abiotic stress reduces the plant defence compounds and increases the digestible proteins leading to their palatability to insect herbivores (19, 24). The drought conditions also increases the concentrations of the secondary metabolites in some plant species (20), thus herbivore performance vary according to taxonomy and feeding behaviour of insect species (24). Hence it is important to understand how the drought conditions influence the plant-insect herbivore interactions from both the plant and the herbivore perspective.

The generation of reactive oxygen species (ROS) is one of the earliest biochemical responses of eukaryotic cells to abiotic stress caused by water loss. When plants are subjected to stress, a variety of ROS are generated, such as superoxide radical (O^{2-}), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH) (15). Increase in protein degradation, lipid peroxidation, DNA fragmentation and ultimately result in cell death due to increased ROS production (5, 34). Plants under normal growth conditions produce low levels of ROS and when subjected to drought, the cellular homeostasis of cells is disrupted and ROS production is enhanced (34). Plants that exhibit tolerance to stress tend to minimize damage to their metabolism by regulating oxidative enzymatic activity, including enzymes that inactivate ROS such as ROS scavenging enzymes superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) (5). This capability can be correlated with the drought resistance of plants.

Piper betle is one such widely growing tropical asian vine cultivated in most of south and Southeast Asia for their heart-shaped leaves which are of commercial importance. It grows best under the shaded, tropical forest ecological conditions with a rainfall of about 2250-4750 mm, relative humidity and temperature ranging from 40-80% and 15-40°C, respectively and thus to an extent relatively more drought sensitive compared to our another model plant Castor. Piperaceae plants are rich in insecticidal piperamides (44) which makes the food consumption of Lepidopteran insects relatively difficult, leading to less consumption of this plant species.

Castor crop is a major non-edible commercial oil seed crop cultivated in semi-arid and arid regions of India, Russia and China. India is the world's largest producer of castor seeds (7). Castor is a drought tolerant crop and grows well in hot climate. A scanty rainfall of about 8 -10 cm per annum is enough for its optimum growth. The lack of water and the increasing of atmospheric temperature have severe impact on the *P. betle* plants and especially quality of the commercial product i.e. its leaves are drastically declined. Water resources are gradually declining year by year and temperatures are rising over the years. Though work has been already reported on the defense strategies of castor plants (43), we are interested in observing a different variety of Castor widely grown in India, "Kranti". This study aimed to (i) Compare the physiological and biochemicals changes under drought on high water demanding *Piper betle* L. (Piperaceae) and less water demanding dry land crop, *Ricinus communis* L., (ii). Understand the comparative defense strategies of both Piper and castor plants under drought and (iii). Effects on the food consumption and feeding performance of *S. litura* on these drought effected plants.

MATERIALS AND METHODS

I. **Chemicals**

Phenolic acids such as p-hydroxybenzoic acid, chlorogenic acid, syringic acid, synapic acid, ellagic acid, ferulic acid, gallic acid and caffeic acid were purchased from Sigma Aldrich (purity~99%) and were used as standards for comparison. The solvents were of HPLC grade and the reagents used for estimations were of 99% purity (Merck chemicals, Darmstadt, Germany). The water used was treated in a Milli-Q water purification system (Millipore, Bedford, MA)

II. Experimental plants

Piper betle L. vines were collected from Central Institute of Medicinal and Aromatic Plants, Hyderabad. In each pot (30 x 40 cm), one *P. betle* cutting was planted. The experimental pots were divided into two sets (control, treated), each set comprised of 10 individual betle plants. Each betle plant received 500 ml water daily in first set of plants (control). Plants in stress treatment were watered before irreversible necrosis occurred (i.e. 7 to 8 days, after the experiment started and then after every 4-5 days during the experiment i.e. they received only 21-31% of water, given to well-watered control plants.

Castor seeds were sown in plastic pots (30×40 cm) at 1 seed per pot. Pots were arranged in completely randomized experimental design. These experimental pots were divided into two sets, each set comprised of 10 pots. Each pot had received 600 ml H_2O every 2 days in case of first set of plants (named as control), whereas, every 10 days in case of second set of experimental pots (named as treated).The collection of fresh leaves from the experimental plants is done when they are 60 d old. These leaves were properly weighed and processed for the estimation of physiological, biochemical and oxidative enzymatic levels in both control and treated plants.

All the plants and were grown at $30\pm2^{o}C$ temperature 60±5% RH, 16:8 L: D photo period and were fertilized with N/P/K = 14:14:14 for supplying major plant nutrients.

III. Maintenance of insect cultures

The start-up culture of *Spodoptera litura* (F.) was raised from egg masses collected in a sweet potato field at the Acharya N.G. Ranga Agricultural University Campus, Rajendranagar, and Hyderabad. Neonate larvae of *S. litura* were kept in plastic tubes (25 cm dia) and provided with semi-synthetic (artificial) diet for successive generations in the insectary at IICT, Hyderabad, under temperature of 26 ± 2°C, RH of 65 ± 5% and photoperiod of 12:12 h (light:dark).

IV. Estimation of chlorophyll content

The chlorophyll concentration of *P. betle* and *R. communis* was determined by adding 10 µL aliquot of the leaf proteins to 1 mL of 80% (v/v) acetone. The aliquot was vortexed and then centrifuged for 2 min at room temperature in a micro centrifuge. The supernatant was collected into a fresh eppendorf tube without disturbing the pellet, and the absorbance was read. Chlorophyll concentration was determined by measuring absorption with a spectrophotometer at 645 and 663 nm (6).

V. Measurement of relative water content (rwc)

Relative water content is considered a measure of plant water status, reflecting the metabolic activity in tissues and used as a most meaningful index for dehydration tolerance. For estimation of relative water content the method described by Shahniyar *et al.* (46) was employed using following formula. RWC=100× (FW-DW)/(TW-DW). Leaves collected from the experimental plants were weighed immediately (fresh weight, FW), and then cut into parts and placed in water in a closed Petri dish. After 24 h at 4°C, leaf pieces were weighed again (turgid weight, TW). The dry weight (DW) was measured after 48 h at 60°C.

VI. Biochemical analysis

Comparative analysis of biochemicals from the drought induced and normal *P. betle* and *R. communis* plant leaves were carried out employing standard methods. Estimation of carbohydrates (13) aminoacids (36) proteins (31) total phenols (50) and flavonoids (39) were done by extracting the bio-chemicals from the leaves excised at the upper end of petiole followed by maceration in a tissue homogenizer and were expressed as µg/g FW.

VII. Phenolic acids extraction and hplc analysis

(i). **Extraction:** 1 g leaf was weighed and phenolic acids extracted in 95% methanol for 3d under continuous shaking condition. The solution filtered and evaporated into dryness by rotary evaporator. The dried material re-suspended in 2mL of HPLC grade methanol and used for colorimetric and HPLC analysis.

(ii). **Analysis:** Phenolic acids were analysed using HPLC according to the method described by Tuzen and Ozdemir (52). The separation of phenolic compounds was accomplished on a Gilson GX-271 semi preparative HPLC system. The column was C_{18} (2.5 x 30 cm Gilson apparatus) and a liquid handler with auto injector was employed. For phenolic acid analysis a gradient elution programme was applied and elution was done with solvent A (acetic acid/water (2:98 v/v)) and solvent B (acetic acid: acetonitrile: water (2:30:68 v/v/v)) as mobile phase. Initial condition was programmed as 100% A; 0-5 min, changed to 100% B; 25-35 min., with a flow rate of 1.0 mL/min and the sample injection volume was 100 µL. The signals were detected at 254 nm. Retention times for the standard compounds and the major peaks in the extract were recorded. Identification and determination of the separated compounds were made by comparison of retention time. All the experiments were performed in five independent replicates.

VIII. Measurement of antioxidative enzymes

For measuring all the antioxidative enzyme activities in water stress effected and normal *P. betle* and *R. communis* plants, the leaf material from the treated and control plants were collected. 1g of the leaf material homogenized using suitable buffer solutions. Standard methods employed to estimate antioxidative enzymes and the methods used are as follows: peroxidase (22), catalase (1) and super oxide dismutase (8) and concentrations are expressed in mU/g FW activity.

IX. Herbivore feeding bioassays

Feeding behaviour and food consumption of *Spodoptera litura* F. on drought effected and normal plants were measured in following types of bioassays.

(i) **No choice experiments**: To study the rate of food consumption by *S. litura* on two test plants-*Piper betle* and *R. communis*, leaves from laboratory grown plants were collected. A small circular disc of 21 cm^2 area was cut from the leaves of each plant. Each leaf disc was transferred to four different Petri plates of 15 cm dia containing moist filter paper. Leaf discs from normal unstressed plants were taken as control. In each Petri dish, healthy third instar larvae of *S. litura* pre-starved for about 3 h were introduced to assess its feeding performance. Progress of consumption of leaf area was measured at 6 h intervals for 24 h in both treated and control leaf discs and the leaf discs were replaced once they were consumed totally by the larvae.

(ii) **Dual choice experiments**: This experiment was performed in similar way as described for no choice experiment, except the larvae were offered a choice of feed. In these experiments two leaf discs of choice were placed in same Petri dish and were provided as food to larvae simultaneously. The following combination of leaf discs were tested in this method: (a) Piper normal leaf vs. piper drought stressed leaf (b) Piper normal leaf vs. castor normal leaf, (c) Piper drought leaf vs. castor drought leaf and (d) Castor normal leaf vs. castor drought stressed leaf

(iii) **Multichoice experiments:** To find the effects of drought stress on feeding preferences of *S. litura*, all four types of plants: (a) piper normal (b) piper drought effected (c) castor normal and (d) castor drought effected leaf discs were offered to larvae simultaneously in single Petri dish. Four leaf discs of 11cm^2 area of each case was cut and placed in one Petri dish measuring 15 cm dia. and the feeding behaviour as well as food consumption of the larvae was recorded for 24h duration. Each experiment was replicated 3 times and average of 10 experimental sets was calculated for mean with standard mean error. Areas of control and stress effected leaf discs consumed were measured after 6 h using an AM-300 leaf area meter (ADC, Bioscientific Limited, England) and herbivore feeding is calculated using following formula:

$$\text{Rate of food consumption / leaf area consumed} = [(C \text{ or } T) / (C+T)] \times 100\%,$$

Where, C: Consumption of control discs, and T: Consumption of treated discs.

Ten experimental sets were assayed in similar manner, where each experiment was replicated thrice. The mean of 10 sets was taken for each case and the percentage of area consumed with standard mean error was calculated.

X. Statistical analysis

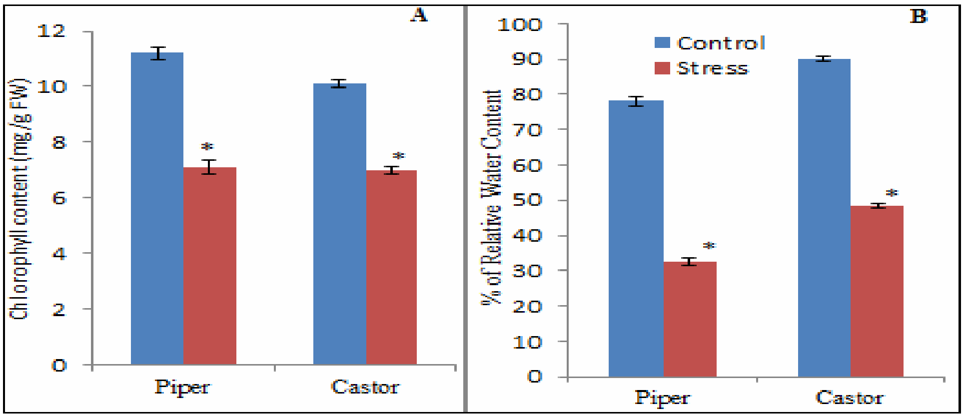
Quantitative differences in biochemical and enzymatic changes of control and treated *P. betle* and *R. communis* plants were measured and compared using t-test. The differences in the phenolic acid levels between water stress induced (water deficit) plants and normal healthy piper and castor plants were calculated from HPLC chromatograms and were analyzed using paired t-test at $P<0.05$. The data were presented as mean±SE. For all feeding bioassays (except dual choice tests) with S. *litura* one-way ANOVA was used and the means were separated using Tukey's HSD. For dual choice assays paired t-test was employed. All statistical analysis was done and the figures were plotted using the software Origin (Ver 8.0).

RESULTS AND DISCUSSION

Physiological response of *P. betle* and *R. communis* to water deficiency

Leaf chlorophyll content provides valuable information about physiological status of plants. Gitelson and Merzlyak, (16) observed that Chlorophyll content tends to decline rapidly when plants are under stress. A significant decrease ($P< 0.05$) was found in chlorophyll concentration (7.45 mg/g FW) of *P. betle* grown under drought stress compared to control plants (11.52 mg/g FW) after 60 d of treatment. Chlorophyll content (7.2 mg/g FW) in the stress effected leaves of *R. communis* were decreased significantly ($P< 0.05$) compared to the control leaves (10.2 mg/g FW) due to water stress after 60d treatment (Figs. 1A). Anjum *et al.* (4) reported reduction in chlorophyll content under drought stress as a sign of oxidative stress. The decrease in chlorophyll content under drought stress has been considered as a typical symptom of oxidative stress and may be the result of chlorophyll degradation. Similar results were reported in sunflower plants due to drought stress (32). Furthermore, reduction in chlorophyll content has been associated with loss of chloroplast membranes, excessive swelling, distortion of the lamellae vesiculation, and the appearance of lipid droplets (27).

Relative Water Content (RWC) is measure of plant water status and reflects the metabolic activity of leaf tissues and used as an important index for dehydration tolerance. Control *P. betle* leaves showed high RWC (78.2%) under normal conditions whereas significant drop in RWC (33.7%) is noticed in *P. betle* plants under drought stress. In control castor plants high RWC (91%) was maintained under normal condition with sufficient water source compared to plants under drought stress which showed striking decrease in percentage of RWC (47%) (Figs. 1B). Plant leaf water status is affected by reduced availability of water, leaf temperature and stomatal opening and closing (4). Normally in photosynthesis, translocation of sugars and growth are the first factors affected by water stress (45). Nayyar and Gupta, (40) recorded that the decrease in the relative water content (RWC) in response to drought stress is seen in wide varieties of plant leaves. Exposure of plants to water-stress substantially decreased the relative water



Figures 1A &1B. Physiological changes, leaf chlorophyll and relative water content of *P. betle* and *R. communis* plants in response to water-stress conditions. Quantitative changes in chlorophyll content (µg/g FW) (A) and RWC (%) (B). Significant differences ($P < 0.05$) are indicated by asterisks (paired t-test) between the control and stress induced plant (N = 20). FW- Fresh Weight of leaves

content with a concomitant increase in leaf temperature (49). Drought stress affects photosynthesis directly by causing changes in plant metabolism or indirectly by limiting the amount of CO_2 available for fixation (30). From the results, it was found that drought resistance of *P. betle* and *R. communis* plants is related to its ability to maintain higher relative water content in the leaves and thus, exhibited clear and obvious responses to abiotic stress caused by water. Though similar reduction pattern was observed in concentration of total chlorophyll content in both species, higher relative water content present in *R. communis* leaves would cater to the metabolic needs more efficiently and confer its drought tolerance ability.

Biochemical responses of *P. betle* and *R. communis* to water deficiency

Statistical analysis showed differences (P<0.001) in biochemical constituents of plants which are affected by the water stress. Drought stress caused significant fall (230.0 µg/g FW) in the carbohydrate content of the *P. betle* compared to control (337.037 µg/g FW) plants (Fig. 2A). A significant decrease (59.2 µg/g FW) was also recorded in the carbohydrate contents of the castor plants due to drought stress in comparison with control (128.1 µg/g FW) plants (Fig. 2A). The carbohydrate content reduction was due to the improper utilization of sugars in the anabolic and catabolic pathways that occurs in plant leaves (14). The percentage increase in carbohydrates over the control in piper and castor was 31.8% and 53.8% respectively. The total protein concentration was decreased in drought effected *P. betle* (140.8 µg/g FW) plant leaves compared to control (246.9 µg/g FW) and in *R. communis* the protein concentration was almost halved (265.7µg/g FW) than control (502.3 µg/g FW), i.e. a decrease of 43.0% and 47.1% respectively over controls (Fig. 2C). Low RWC and excess loss of water led to perceptible changes in interference of leaf RWC on total soluble carbohydrates and proteins content.

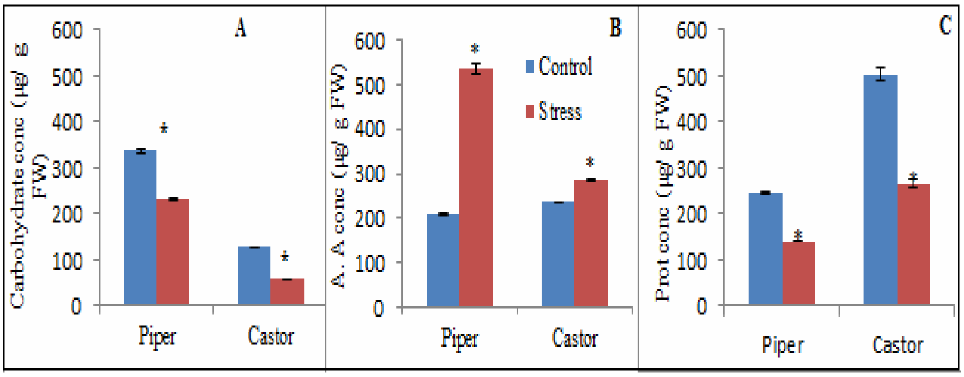
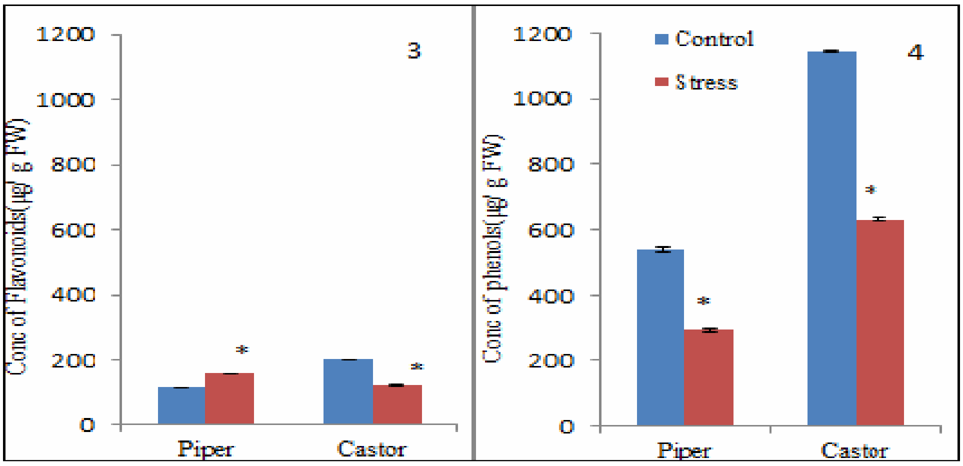


Figure 2 A, B and C. Quantitative changes in the leaf biochemicals, carbohydrates, amino acids and proteins (µg/g FW) of *P. betle* and *R. communis* due to water stress conditions. Bars indicate the biochemical content (Mean ± SE). Mean values (N = 20) of the control and stress induced plant biochemicals followed by asterisks are significantly different. Student t-test used and P < 0.001. g FW- Gram Fresh Weight of leaves.

Abiotic stress caused decrease in protein content in the water stress affected piper and castor plants compared to control plants. It may be due to hydrolysis of proteins brought out by certain amides or amino acids present inside the cell which use protease as their substrate. Abiotic stress also increased the free amino acid in both piper and castor bean plants under drought conditions (536.22 µg/g FW and 285.2 µg/g FW) than control (210.1 and 235.7 µg/g FW) plants i.e. a decrease of 155.2 and 21.0% respectively (Fig. 2B, Fig. 7). The proteins under abiotic stress conditions will break down due to certain amides or certain amino acids present inside the cell. They will use protease as their substrate and ultimately brings the hydrolysis. The accumulation of amino acid under stress in many plant species has been correlated with stress tolerance, and its concentration was higher in stress-tolerant than in stress-sensitive plants (51). Amount of amino acids present in a cell influence protein solvation and preserve the quaternary structure of complex proteins. It also maintains membrane integrity under dehydration stress and reduces oxidation of lipid membranes or photo-inhibition (11). The enhanced amino acid content under stress could help in the maintenance of energy fluxes of the chloroplast and also they will interact with other enzymes present in the cell in stabilizing their structure and plays key role in the tolerance of severe drought conditions. Yadav *et al.* (55) reported the increased amino acid content in chick pea under drought stress is due to hydrolysis of proteins.

The water stress in *P. betle* plants also caused a significant ($P < 0.001$) increase in the flavonoid content (38.4%) and a decrease in phenolic contents (37.7%), (Fig-7) in comparison with control or healthy plants (Figs. 3 and 4). The flavonoid contents increased (159.4 µg QE/g FW) in water-stress induced plants than control (115.2 µg QE/g FW) which protects the plants against UV irradiation and decreased in phenol content (292.5 µg GE/g FW) than control (539.0 µg GE/g FW) whereas flavonoid content decreased (124.6 µg QE/g FW) in *R. communis* plants than control (200 µg QE/g FW) which is contrary to the pattern observed in the piper plants (Fig. 6). The phenols also



Figures 3 and 4. Quantitative changes in the leaf secondary metabolites, flavonoids (µg QE/g FW) and total phenols (µg GE/g FW) of *P. betle* and *R. communis* due to water stress conditions. Bars indicate the biochemical content (Mean ± SE). The comparision of biochemical contents between the control and stress induced plant biochemicals (N = 20). g FW- Gram Fresh Weight of leaves. QE- Quercitin Equivalents. GE- Gallic acid equivalents. Mean values followed by asterisks are significantly different. Student t-test used and $P < 0.001$.

decreased (630.6 µg GE/g FW) in stress affected *R. communis* plants compared to healthy plants (1144.9 µg GE/g FW) (Fig. 4) and the percentage decrease over control was 44.9% (Fig.7).

Biochemical changes in secondary metabolites

Changes in primary metabolites may change the efficacy of secondary metabolites (28). Quantitative analysis of the phenolics in both plants had significant variation among the treated and normal healthy plants. A significant reduction of chlorogenic (137.0 µg /g FW), caffeic acid (28.1 µg/g FW), gallic acid (109.5 µg/g FW), sinapic acid (89.6 µg/g FW), syringic (67.8 µg/g FW), ferulic (47.0 µg/g FW) and ellagic acid (44.0 µg/g FW) noticed under drought stress in *P. betle* plant leaves than their normal counterparts. A significant reduction of chlorogenic (13.9 µg/g FW), caffeic (5.2 µg/g FW), syringic (2.9 µg/g FW), ferulic (10.8 µg/g FW) and *p*-hydroxybenzoic acids (9.3 µg/g FW) were recorded in the castor plant leaves due to water stress, when compared with control plants (18.6, 10.5, 4.4, 15.2 and 12.9 µg/g FW) respectively (Fig. 5). The quantity of foliar phenolic compounds were high in control *P. betle* plants and decreased significantly in affected leaves of *P. betle* due to the breakdown of the chlorophyll and phenolic pigments under drought conditions. Hura *et al.* (25) reported reduction in concentration of ferulic acid in *Zea mays* L. due to effect on chlorophyll apparatus and phenolic pigments. Similar results were reported by Ahmed *et al.* (2) that the reduced phenolic content in cotton leaves at ball formation is due to increased leaf senescence and

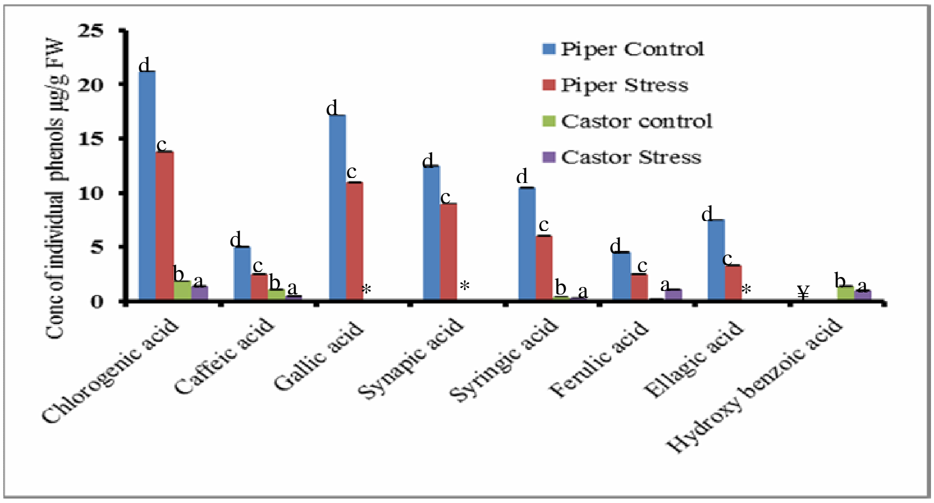


Figure 5. Quantitative changes of individual phenolic compounds (µg/g FW) in *P. betle* and *R. communis* due to water stress. Comparative individual phenol contents of *P. betle* and *R. communis* in water stress affected plants (paired t-test) between the control and stress induced plants (N = 3). GE- Gallic acid equivalents. FW- Fresh Weight of leaves. *- phenolic acid is not detected in Castor plants. ¥ - phenols not detected in *Piper* plants. Mean values followed by different alphabets are significantly different for individual phenols. Student t-test used and $P < 0.05$.

degeneration of photosynthetic pigments. The results indicate that the degradation of photosynthetic pigments in betle and castor leaves under water deficiency stress condition results in the reduction of phenolic compounds.

Oxidative response of *P. betle* and *R. communis* plants to water deficiency

Apel and Hirt, (5) reported that in order to minimize the effects of oxidative stress, plants have evolved a ROS scavenging enzyme such as, SOD, POD and CAT. These antioxidant enzymes have capability to scavenge ROS and reduce the damage. The production of higher level of these oxidative enzymes may contribute to tolerance under drought conditions caused by oxidative damage (47). The balance between ROS production and activities of antioxidative enzymes determine whether oxidative signalling and/or damage will occur (35). Peroxidases play a key role in scavenging H_2O_2, which is produced during dismutation of O^{2-} in other enzymatic reaction catalyzed by superoxide dismutase (21). These peroxidases decompose the toxic H_2O_2 by using certain phenolic compounds and/or antioxidants as their substrates (41). These peroxidases are major cellular scavengers of H_2O_2 and the removal of these compounds is very essential for the plant cell to avoid inhibition of enzymes that are useful in the calvin cycle of photosynthesis. Under drought stress *P. betle* leaves, POD activity increased due to increase of oxidative stress in plant cells. Similar results are also reported in wheat plants under water stress induction which produced several internal protective defensive oxidative enzymes as a part of induced defense mechanism (23), which is enough to avoid

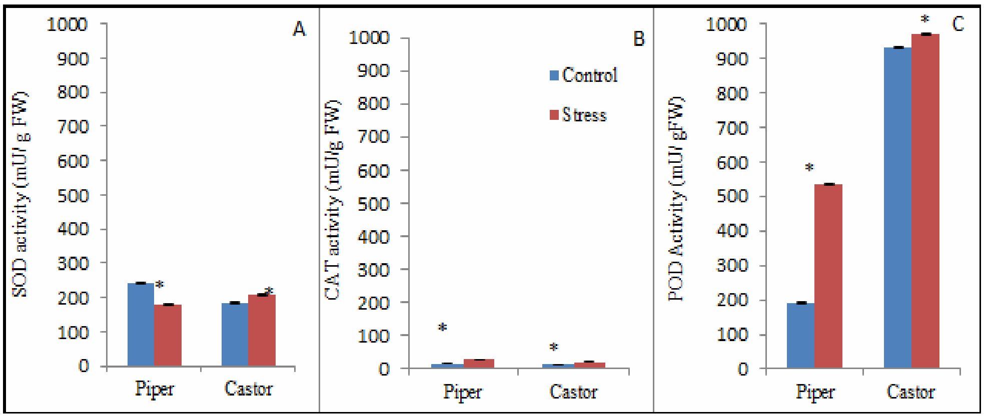


Figure 6 A, B & C. Defensive enzyme activities of leaf extract from *P. betle* and *R. communis* plants due to water stress. Superoxide dismutase (SOD), Catalase (CAT) and Peroxidase (POD) activities (mU/g FW of protein). Enzyme activities of are observed in Stress induced and control plants of both *P. betle* and *R. communis* plants (paired t-test) (N = 20). Mean values followed by asterisks are significantly different. Student t-test used and $P < 0.001$.

injuries of active oxygen species. *P. betle* leaves had increase in POD activities (536.5 mU/g FW) under drought condition suggesting that it has high ROS scavenging capacity and this helps in less oxidative damage to the plant. Whereas, in control plants POD activity (192.5 mU/g FW) (Fig. 6C) is very less since there is no ROS generation or oxidative stress. A significant increase in POD activity is observed (969.8 mU/g FW) in *R. communis* leaves under drought stress compared to control (932.5 mU/g FW) (Fig. 6C) which reflects it has high ROS scavenging capacity and help in decreased oxidative damage to the plant.

SOD acts as the first line of defense in response to any type of plant stress. It is the major scavenger of O^{2-} and its enzymatic action results in the formation of H_2O_2 and O^2. It is evident from the present results, that the increased content of SOD decreases the ROS levels, ultimately leading to the plant survival. The activity of SOD in the leaves of *P. betle* plants increased (241.2 mU/g FW) under drought stress compared to control (178 mU/g FW) plants (Fig. 7A) In the present report, the activity of SOD was found to increase (208.3 mU/g FW) in leaves of castor plants under water-stress in comparison with control (183.2 mU/g FW) plants (Fig. 6A). Also there was a considerable increase in the CAT activity (26.7 mU/g FW) noticed in *P. betle* leaves under these conditions than control (14.4 mU/g FW). There was considerable increase in the CAT activity (19.4 mU/g FW) in stress affected *R. communis* leaves when than control (12.8 mU/g FW) (Fig. 6B). CAT is main enzyme to eliminate H_2O_2 in the mitochondria and microbody and thus help in reducing the detrimental effects of oxidative stress (48). In water stressed piper plants, only SOD was inhibited (26.1%), but SOD was stimulated in castor plants (13.7%), while, CAT activity in piper and castor water stressed plants was stimulated by 85.42% and 51.56% respectively. POD activities were also stimulated in both piper and castor plants by 51.56% and 4% respectively.

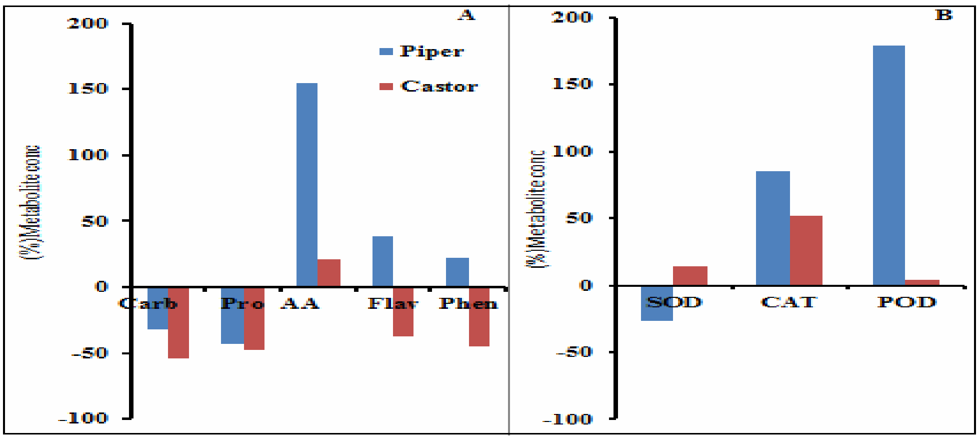


Figure 7A & B. Percentage change in metabolite concentration in water stress affected *P. betle* and *R. communis* over control and inhibition (-)/ Stimulation (+) of Defensive enzymes over control in drought stressed plants. Carb- Carbohydrates, Pro- Proteins, AA- Amino Acids, Flav- Flavanoids, Phen- Phenols, SOD- Superoxide dismutase, CAT- Catalase and POD- Peroxidase.

Increase in flavonoid and phenol concentration may render the resistance to the betle plants to cope up with the drought conditions. At the same time amino acid concentration was increased and decrease in protein concentration was observed. Hydrolysis of more proteins indicate that useful proteins like RUBISCO, a main component of chlorophyll apparatus in leaves are hydrolysed, decreasing the ability to synthesize carbohydrates which further lead to the reduced amounts of sugars produced. Significantly increased activities of SOD, POD and CAT in the present investigation suggested a higher antioxidative ability, of *P. betle* plants to water stress. Its considerable tolerance is attributed mainly to POD which was drastically increased compared to other oxidative enzymes. Hence, all these conjugated parameters are helping *P. betle* plants to survive the water stress. Compared to betle plants, better resistance of castor plants is likely due to its higher ability in maintaining the viability of leaves by retaining more RWC, higher amounts of proteins stored and of phenols produced (Figs. 1B, Table 1). Lower level of decreased protein concentration in castor leaves in comparison to betle leaves relates to the more protection of chlorophyll protein RUBISCO. All the three oxidative enzymes under study were increased that conferred resistance to drought among which POD production were induced in much higher amounts. In both plants, POD was highly induced which indicate that POD may be key regulator in drought tolerance or resistance (Table 2). Thus *P. betle* and *R. communis* plants have defensive mechanisms such as, physiological, biochemical and oxidative responses to survive water stress and increase the overall plant resistance to stress conditions.

Impact of water stress on feeding of herbivore insect

Petri-dish no-choice and choice bioassays are important tools to study the impact

Table 1. Quantitative changes in concentrations of primary and secondary metabolites in water stress induced and control *P. betle* and *R. communis* plants

Metabolites (µg/ FW)	*Piper betle* L.		*Ricinus communis L.*	
	Control	Stress	Control	Stress
Carbohydrates	337.0±3.1	230.0±2.0*	128.1±0.6	59.2±0.6*
Proteins	246.9±1.8	140.8±1.1*	502.3±11.7	265.7±8.0*
Amino acids	210.1±2.2	536.2±10.9*	235.7±1.1	285.2±3.3*
Flavonoids	115.2±0.8	159.4±1.3*	200.0±0	124.6±2.1*
Phenols	239.0±6.1	292.0±2.5*	1144.0±5.0	630.6±5.0*

Mean values ± SE followed by asterisk in a row are significantly different. Student t-test used and $P < 0.001$

Table 2. Differences in enzyme activities of water stress induced *P. betle* and *R. communis* plants in comparison with control plants

Enzymes activity (mU/g FW)	*Piper betle* L.		*Ricinus communis L.*	
	Control	Stress	Control	Stress
Superoxide dismutase	241.0±0.9	178.0±0.9*	183.2±1.2	208.3±1.0*
Catalase	14.4±0	26.7±0.1*	12.8±0.1	19.4±0.1*
Peroxidase	192.5± 1.4	536.5±1.9*	932.5±1.3	969.8±0.9*

Mean values ± SE followed by asterisk in a row are significantly different. Student t-test used and $P < 0.001$

of herbivory on different plants and these assays normally correlates well with bioassays done using whole plants and also with measures of resistance in field (18). To evaluate the insect feeding and other responses on the *P. betle* as well *R. communis* under drought stress, a polyphagous agricultural pest, *S. litura* larvae were used. In no-choice experiment, 64.5 ± 0.194 % area of castor stress affected leaf discs were fed to *S. litura,* which was more compared to feeding on normal leafs (54.8% ± 0.283 %). In experiments, when piper discs were provided as food, less than 25% feeding was observed with both normal and drought stressed leaves. All mean values were significantly different at $P = <0.001$. Results were consistent for no choice, dual choice and multiple choice tests, where drought stressed plant leaves were more preferred than normal control plants (Table 3). In dual choice experiments, *S. litura* preferred the drought stressed piper leaves (22.6 ± 0.245 %) than normal unaffected leaves (19.6 ± 0.345 %). When given a choice between piper normal leaf and castor normal leaf, larvae fed largely on castor normal (74. 3 ± 0.214%) than on piper normal (18.6 ± 0.245%). Choice given between piper drought leaf and castor drought leaf showed more feeding performance on castor drought leaf (65.7 ± 0.186 %) than on piper drought leaf discs (23.6 ± 0.156%) and in dual choice made between castor normal leaf and castor drought stressed leaf, *S. litura* preferred the drought stressed leaf discs (54.7 ± 0.235%) than normal control leaf discs (42.8 ± 0.253%). All mean values were significantly different at ($P < 0.001$ when analysed by paired t- test). In multi choice petri-dish assays, when all the four types of leaf discs were offered as choice, the larvae preferred the drought affected castor (34.0 ± 0.345%) than normal castor leaf (27.9 ± 0.197%), which was higher than piper drought effected leaf discs (17.4 ± 0.945 %) and least preferred choice were the piper normal plant leaf discs (12.5 ± 0.345%). All values were analysed using one way- ANOVA and means were separated using Tukey's HSD.

Table 3. Results of feeding behaviour and food consumption of *Spodoptera litura* F. on water stress induced *P. betle* and *R. communis* measured in various bioassays

S.No.	Choice assay	Piper		Castor	
		Normal (PN)	Drought stress (PDS)	Normal (CN)	Drought Stress (CDS)
1	No- Choice	20.4 ± 0.296%^{a}	24.53 ± 0.374%^{a}	54.8 ± 0.283 %^{a}	64.4 ± 0.194^{a}
2	Dual Choice				
A	PN Vs PDS	19.6 ± 0.345 %^{b}	22.6 ± 0.245 %^{b}	-	-
B	PN Vs CN	18.6 ± 0.245%^{c}	-	74. 3 ± 0.214%^{c}	-
C	PDS Vs CDS	-	23.6 ± 0.156%^{d}	-	65.7 ± 0.186 %^{d}
D	CN Vs CDS	-	-	42.8 ± 0.253%^{e}	54.7 ± 0.235%^{e}
3	Multiple Choice	12.5 ± 0.345%^{f}	17.4 ± 0.945 %^{f}	27.9 ± 0.197%^{f}	34.0 ± 0.345%^{f}

Mean values ± SE followed by alphabets in a row are significantly different at $P < 0.001$. (Student t-test is used for Dual Choice and one-way ANOVA for No-choice and Multiple choice.

Piper plants (rich in total and individual phenol concentrations) were not preferred by *S. litura* compared to castor plants. It was also observed that nutrient~~s~~ quality differences in normal control plants were also not large to make it preferred for feeding by *S. litura*, though it exhibited better performance on drought effected piper plants than normal piper plants. The differential feeding of this insect to stressed plants could be explained from decreased secondary metabolites such as phenols and increased palatability due to nutritional quality changes of the drought stressed plants (19, 24). In our previous study, phenolic acids (chlorogenic acid, caffeic acid and syringic acid) acted as strong antifeedants to *S. litura*. When individual phenolic acids were studied under drought conditions, the concentrations of these phenols were drastically reduced in *R. communis* plants than in *P. betle* plants (Fig. 5). These results indicated that reduction in phenols contents correlates with the increased feeding of *S. litura*, which was higher on drought stressed castor plants (53). Similar results were observed when *Alliariam petiolata* (Brassicaceae) were fed with *S. litura* where the feeding was high in severely drought affected plants due to decrease in amount of defence compounds (19). The feeding of drought stressed *Holcus lanatus* to *S. litura*, prolonged the larval duration, increased pupal weight and higher adult eclosion rates (54). The generalist aphid *Myzus persicae* (Sulzer) fed on water stressed *Arabidopsis thaliana* (L.) plants, caused metabolite concentrations in the phloem sap and growth was highest on plants under drought stress conditions (33). The warming associated with drought conditions reduces the levels of phenolics and increase the levels of terpenoids in plant foliage (24)**.** The phenolic glycosides concentrations were inversely related to the larval development of gypsy moths (*Lymantria dispar*) feeding on aspen (12). Thus exposures to drought conditions influenced the plant-insect interactions by changing plant's foliar chemical constituents.

CONCLUSIONS

The drought resistance of *P. betle* and *R. communis* plants is related to its ability to maintain higher relative water content in the leaves. Though similar reduction pattern is observed in concentration of total chlorophyll content in both the plant species, higher relative water content present in *R. communis* leaves might cater to the metabolic needs more efficiently and confer it drought tolerance. Accumulation of antioxidants and free amino acids along with decrease in phenols is observed and these changes in leaf biochemistry in response to stress are a significant shift in the nutritional quality of leaf for herbivores. In drought stressed castor plants, the main anti-feedant compounds, i.e. phenols were drastically reduced, besides exhibiting better retention of water levels and increased soluble carbohydrates, thus making it more suitable for pest feeding compared to Piper plants and also than plants grown in normal conditions.

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