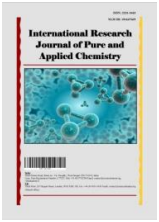

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Role of Ascorbate–Glutathione (AsA–GSH) Pathways in *Phytophthora* Leaf Blight Disease Resistance in Taro (*Colocasia esculenta* L. Schott)

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Authors' contributions

This work was carried out in collaboration among all authors. Authors YID and MD carried out the experiment, data analyses, interpretation and writing of the draft manuscript. Authors JM and MRS Conceptualize the research, data analyses and write, review and edit. All the authors reviewed and approved the content of the manuscript.

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ABSTRACT

Taro (*Colocasia esculenta* L. Schott) is one of the important staple vegetable crops grown worldwide for its nutritious corms, leaves, and pseudostems. Taro invaded by leaf blight disease caused by *Phytophthora colocasiae* Racib. (*Pc*) resulted in 50% yield loss. On the other hand, inherent defense mechanisms of taro encounter the invaders to protect the plant from *Pc* invasion. The ascorbate–glutathione (AsA–GSH) pathways play an essential role in scavenging reactive oxygen species (ROS), a common phenomenon in plant–pathogen interaction. The present study focused on AsA–GSH regulations of thirty genotypes of taro under induced *Pc* infection. RCMC–5, among the tested taro genotypes, registered consistently higher induction of AsA, GSH, Ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR), and dehydroascorbate reductase (DHAR) to encounter the *Pc* infection and overproduction of ROS. However, glutathione reductase (GR) was more prominent in DP–25, Jhankri, and TSL. AsA–GSH encounters the ROS overproduction, which was confirmed with lower H_2O_2 (0.20 µM g^{-1} FW) and malondialdehyde (MDA) content (20.10 nM g^{-1} FW) in resistant genotypes (RCMC–5) subsequently resulted in

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lower *Pc* infection (spot diameter, <2.0 cm and sporangia, <2). RCMC–5 could be one of the lines of interest in taro breeding programs for developing *Pc* resistant lines. AsA–GSH cycle could be a reliable parameter while selecting resistant lines for augmenting breeding strategies in taro against *Phytophthora*.

Keywords: Antioxidants; AsA–GSH cycle; lipid peroxidation; Phytophthora colocasiae; reactive oxygen species; taro.

1. INTRODUCTION

Taro (*Colocasia esculenta* L. Schott) ranks fourteenth among the vegetable crops grown worldwide [1]. Northeastern hill (NEH) region of India, Indo–Burma region in particular, is recognized as the secondary place of origin of taro [2]. Taro plants as a whole, including leaves, pseudostems, and corms, are consumed as vegetables by the tribal communities of this region. Corms and cormels of taro are among the cheapest sources of vitamins, minerals, and dietary carbohydrates for the resource–poor farmers [3]. The productivity of taro is severely affected due to biotic and abiotic stresses. Among the biotic stress, taro leaf blight disease caused by the fungal pathogen *Phytophthora colocasiae* Racib. caused yield loss to the tune of 50% [4]. Understanding host resistance and selecting resistant varieties is an alternative to combat this severe disease as spraying of fungicides is not possible on the waxy leaves. Selection of resistant lines indigenous to NEH region of India would be useful to prevent perceived yield loss occurred due to this severe disease.

Host resistance in plants includes avoidance and tolerance mechanisms to combat invading pathogens attack. Avoidance mechanisms involve morphological and physiological adaptations; however, tolerance mechanisms are associated with a better biochemical and antioxidative system. The involvement of antioxidative enzymes in disease resistance in various crops is well documented [5]; however, limited reports are available on the role of the antioxidants in taro leaf blight resistance [6]. The antioxidants (enzymatic and non–enzymatic) help in scavenging the overproduction of reactive oxygen species (ROS) [7]. Ascorbate–glutathione (AsA–GSH) cycle, also known as the Asada–Halliwell pathway, includes a group of non–enzymatic and enzymatic cellular redox buffers which acts in a concert to equilibrate the ROS owing to stress [8]. The cycle comprises two non–enzymatic antioxidants such as, ascorbate (AsA), glutathione (GSH), and four enzymatic antioxidants, ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR).

ROS in the term of toxic H_2O_2 produced upon the occurrence of stress and lipid peroxidation (MDA content) is one of the hallmarks of ROS led oxidative damage to the membrane [9]. AsA–GSH cycle plays an essential role in inhibiting the overproduction of ROS in many plants [10]; however, reports on AsA–GSH regulations on *Phytophthora* leaf blight disease resistance in taro are unavailable. Hence, we have taken an approach to understand the role of AsA–GSH cycle in the taro–*Phytophthora* pathosystem in the present study. The current investigation focused on the characterization of thirty genotypes of taro indigenous to NEH region of India for *Phytophthora* leaf blight resistance assessing the regulation of AsA, GSH, APX, MDAR, DHAR, and GR involved in AsA–GSH pathways.

2. MATERIALS AND METHODS

2.1 Plant Materials and Experimental Design

The experiment was conducted at Indian Council of Agricultural Research (ICAR) Research Complex for North Eastern Hill Region (ICAR RC NEHR), Manipur, India. The centre is located at 24°50'N latitude, 93°55'E longitude at an altitude of 860 m above mean sea level. A genetic base of 101 genotypes of taro were collected mostly from NEH region of India and from Regional Centre of Central Tuber Crops Research Institute, Bhubaneswar and maintained at ICAR RC NEHR, Manipur. Thirty genotypes of taro comprising of various degrees of resistance following a preliminarily screening as described by Dey et al. (1993) [11] were selected for this study. The details of the selected genotypes were mentioned in the Table 1.

Taro cormels (30–40 g each of thirty genotypes) were planted in pots of size 30×30×30 cm

containing garden soil, sand and farmyard manure (1:1:1). The pots were irrigated at 7–10 days intervals. At 45 days of planting, fully opened leaf discs (8 cm diameter) were collected from all 30 genotypes of taro and placed in petri dishes with moist Whatman paper no. 1. *Pc* spores were collected from the infected leaves in 5 ml of sterile water with the help of a paint brush and the elliptical sporangia were examined on a haemocytometer under microscope at 10x to maintain the spore count of 15000 mL^{-1}. For molecular detection of *P. colocasiae*, 10 µL of spore suspension was inoculated in potato dextrose agar (PDA) medium and the mycelia mats were harvested at 7 DAI. Molecular identification of *Pc* was carried out using the internal transcribed spacer (ITS) region of rDNA amplified using the ITSI (5'TCCGTAGGTGAACCTGCGG 3') and ITS4 primers (5' TTCCTCCGCTTATTGATATGC 3') as described by White et al. (1990) [12]. The specific primers i.e., ITS1 and ITS4 amplified an expected band of 784 bp.

Ten microliters of *Pc* spore suspension (15000 mL^{-1}) were inoculated on the leaf discs and incubated for 96 h at room temperature. Appearance of disease spot and number of sporangia produce per microscopic field was observed at 24 h intervals upto 96 h as described by Dey et al. (1993) [11]. Observations on ROS accumulation (H_2O_2 content and lipid peroxidation by MDA content) along with the ROS scavengers involved in AsA–GSH cycle was observed upon *Pc* occurrence at 48 h. The experiment was repeated three times with duplicate determinations in a completely randomized design (CRD).

2.2 Determination of H_2O_2 and MDA Content

Assay of H_2O_2 content was carried out following the method of Velikova (2000) [13]. Leaf samples were homogenized in 0.1% trichloroacetic acid (TCA) in 1:10 (w/v) ratio and centrifuged at 12,000 g for 15 min at 4°C in a cooling centrifuge. About 0.5 ml of supernatant was mixed with 1 ml of 1 M potassium iodide solution and incubated for 5 min. The oxidation product was measured at 390 nm in a UV-Vis spectrophotometer (Thermo Fisher Scientific, USA).

Lipid peroxidation (MDA content) was determined by estimating the malondialdehyde (MDA) content according to the method of Buege and Aust (1978) [14]. One g of the sample was homogenized in 5 ml of 0.6% (v/v) 2-thiobarbituric acid (TBA solution) in 10% (v/v) TCA and was centrifuged at 12,000 g for 15 min. The supernatant was heated in a boiling water bath for 15 min and cooled in an ice bath. The resulting mixture was centrifuged at 12,000 g for 15 min, and the absorbance was measured at 532 nm. Unspecific turbidity in measurements was corrected by subtracting the reading at 600 nm. MDA concentrations were calculated by means of an extinction coefficient of 155 (mmol L^{-1} cm^{-1}) [15].

2.3 Determination of AsA–GSH Pathways

2.3.1 Ascorbate content (AsA)

Ascorbate content was determined colorimetrically according to Mukherjee and Choudhuri (1983) [16] with modification. AsA was extracted from 0.2 g of fresh leaf samples with 5 ml of 6% TCA. Two ml of the extract was mixed with 1 ml of 2% dinitrophenylhydrazine (in acidic medium) followed by the addition of one drop 10% thiourea (in 70% ethanol). The mixture was then boiled for 20 min in a water bath. After cooling to room temperature, 5 ml of 80% (v/v) H_2SO_4 was added to the mixture at 0 °C (in an ice bath). The absorbance was recorded at 530 nm and the concentration of AsA was calculated from a standard curve plotted with a known concentration of ascorbic acid and expressed as mg g^{-1} fresh weight (FW).

2.3.2 Glutathione content (GSH)

GSH was measured by its reaction with DTNB (5,5′–dithiobis–2–nitrobenzoic acid) solution; to give a yellow coloured product, following the protocol of Moron et al. (1979) [17]. The sample (0.5 g) each was homogenized in 2.5 ml of 5% TCA. The homogenate was immediately acidified by adding 125 µl of 25% TCA and was centrifuged at 10000 rpm for 10 min. The homogenate was cooled on ice, where 0.1 ml of the supernatant was taken and made up to 1 ml with 0.2 M sodium phosphate buffer (pH 8.0). About 2 ml of freshly prepared DTNB solution (Ellman's reagent) was added and the intensity of the yellow colour formed was read at 412 nm after 10 min. A standard curve of GSH was prepared using concentrations ranging from 2–10 nano moles of GSH. The values were expressed as µM g^{-1} of the sample.

Table 1. List of taro genotypes selected for AsA-GSH pathways studies

Sl. No.	Genotypes	Place of Collection	Disease incidence
1	RCMC-1	ICAR, Manipur	MS
2	RCMC-2	ICAR, Manipur	MR
3	RCMC-3	ICAR, Manipur	MS
4	RCMC-4	ICAR, Manipur	MS
5	RCMC-5	ICAR, Manipur	R
6	RCMC-6	ICAR, Manipur	MR
7	RCMC-7	ICAR, Manipur	MR
8	RCMC-8	ICAR, Manipur	MS
9	RCMC-9	ICAR, Manipur	MS
10	RCMC-10	ICAR, Manipur	MR
11	Chandel	Chandel, Manipur	MR
12	Kasom Khullen	Ukhrul, Manipur	MR
13	Senapati	Senapati,Manipur	MS
14	Chingpal-2	Manipur	MS
15	Rosentanu	Manipur	MS
16	Balpi Chandel	Chandel, Manipur	MS
17	Jerumpan	Chandel,Manipur	S
18	Parsonu Chandel	Chandel, Manipur	MR
19	Chingpal-1	Manipur	MS
20	Thoubal	Thoubal, Manipur	MS
21	Duradim	Meghalaya	R
22	Topi	CTCRI, Bhubaneswar	MR
23	Satasankha	CTCRI, Bhubaneswar	S
24	TSL	CTCRI, Bhubaneswar	R
25	BBSR	CTCRI, Bhubaneswar	MR
26	BBSL	CTCRI, Bhubaneswar	R
27	DP-25	CTCRI, Bhubaneswar	R
28	Jhankri	CTCRI, Bhubaneswar	MR
29	Muktakashi	CTCRI, Bhubaneswar	R
30	TK-1	ICAR, Manipur	MR

R: Resistant; MR: Moderately resistant; MS: Moderately susceptible; S: Susceptible

2.3.3 Determination of antioxidative enzymes

The control and infected leaf samples (0.25 g) were homogenized with liquid nitrogen in a pre–chilled mortar and pestle and 2.5 ml of extraction buffer containing 50 mM NaPO4 buffer (pH 7.8), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% Triton X–100, 1 mM ascorbate and 10% sorbitol was added. The samples were centrifuged at 15,000 rpm at 4 ^{o}C for 20 min. The supernatant was used for all the enzyme assays.

APX (EC 1.11.1.1) activity was assayed according to Nakano and Asada (1981) [18], measured by the decrease in absorbance due to ascorbate oxidation at 290 nm for 1 min. MDAR (EC 1.6.5.4) activity was assayed by determining the reduction in absorbance at 340 nm according to Hossain and Asada (1984) [19]. Dehydroascorbate reductase (DHAR; EC 1.8.5.1) activity was determined by measuring the increase in absorbance at 290 nm as described by Nakano and Asada (1981) [18]. Glutathione reductase (GR; EC 1.6.4.2) activity was assayed following the methodology of Cakmak et al. (1993) [20]. The oxidation of NADPH was monitored by the decrease in absorbance at 340 nm for 2 min [20].

2.4 Data Analyses

Analysis of variance (ANOVA) was performed to determine significant differences among genotypes for the studied parameters. Heat map depicting the disease incidence, ROS accumulation, and AsA–GSH pathways in the thirty genotypes of taro were estimated using XLSTAT statistical software (XLSTAT Premium 2020.2.1, Adinsoft, NY).

3. RESULTS AND DISCUSSION

3.1 *In vitro* Pc Incidence

Significant variations in disease incidence traits such as spot diameter and the number of

sporangia produced per microscopic field at 24 h intervals. At 96 h, the spot diameter was observed in the range of 1.8 cm to 4.1 cm. Spot diameter was less than 2 cm in the genotypes, DP–25 (1.83 cm), RCMC–5 (1.90 cm), and Kasom Khullen (1.93 cm), and was higher in Satasankha (4.10 cm). Similarly, the number of sporangia per microscopic field was less than 2 in RCMC–5 (1.8) followed by 2.8 in DP–25, 3.3 in Duradim, and 4.3 in Kasom Khullen (Fig. 1). Satasankha possessed higher sporangia (22 numbers). RCMC–5 registered as resistance to *Pc* with less incidence (<2.0 cm), and sporangia count less than two as per the scale derived by Dey et al. (1993) [11]. Nath et al. (2013) [21] reported that the *in vitro Pc*–resistance studies are more authenticated over weather biased *in vivo* studies. The occurrence of leaf spot and sporangia count in the resistant genotypes was low, probably due to their inherent defense system.

3.2 H_2O_2 and MDA Content

H_2O_2 accumulation and MDA content were varied significantly among the tested genotypes under *Pc* infestation (Fig. 2). H_2O_2 content was ranged between 0.20–0.87 µM g^{-1} FW among the tested genotypes. Accumulation of H_2O_2 was lower in the genotypes RCMC–5 (0.20 µM g^{-1} FW), Topi (0.33 µM g^{-1} FW), DP–25 (0.33 µM g^{-1} FW) and TSL (0.34 µM g^{-1} FW) which was as high as 0.81 µM g^{-1} FW in Jerumpan and 0.87 µM g^{-1} FW in Satasankha.

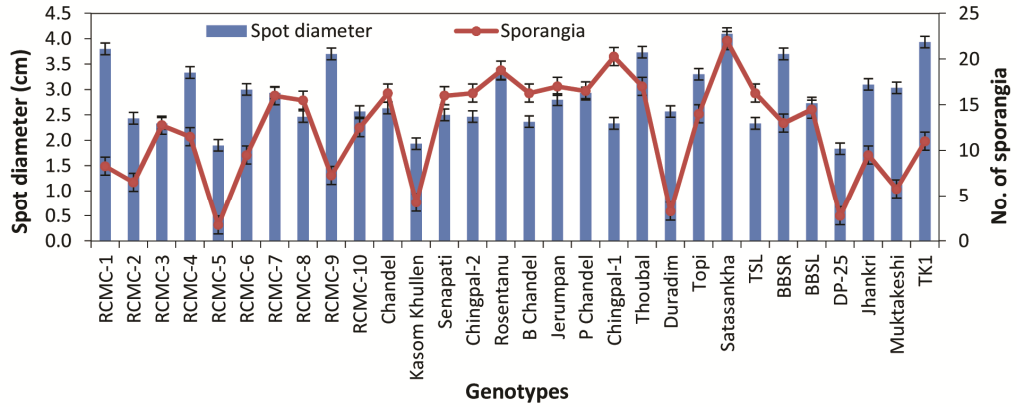

Fig. 1. Spot diameter (cm) and number of sporangia produced in leaf tissues of thirty genotypes of taro owing to *Phytophthora colocasiae* infection. Values are the mean of three replicates and bars represent standard error of means

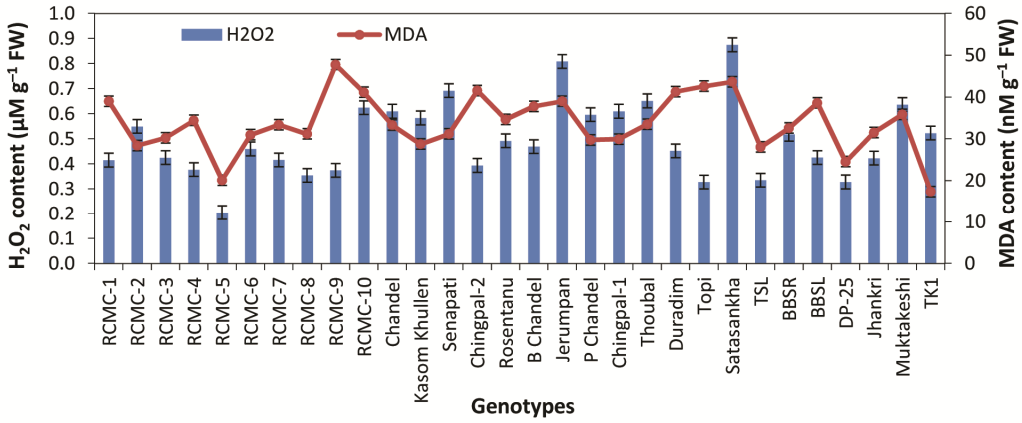

Fig. 2. H_2O_2 and MDA content in leaf tissues of thirty genotypes of taro owing to *Phytophthora colocasiae* infection. Values are the mean of three replicates and bars represent standard error of means

MDA was estimated to be 17.35–47.75 nM g^{-1} FW among the taro genotypes tested for *Pc* infestation (Fig. 2). In the present study, lower lipid peroxidation was observed in TK–1 (17.35 nM g^{-1} FW) followed by RCMC–5 (20.10 nM g^{-1} FW) and TSL (24.54 nM g^{-1} FW) and higher MDA was exhibited in RCMC–9 (47.75 nM g^{-1} FW) and Satasankha (43.66 nM g^{-1} FW). H_2O_2 and MDA are reported to be one of the critical stress markers in plants, which accumulated highly in susceptible lines than resistant [22].

The heat map (Fig. 3) revealed a significant difference in the disease incidence, ROS accumulation, and lipid peroxidation among thirty genotypes of taro tested for *Pc* resistance. It showed a substantial relationship among the *Pc* incidence with the occurrence of H_2O_2 and MDA content. ROS occurrence was lower in resistant genotypes than the susceptible lines under *Phytophthora* infestation [23]. The tested genotypes were grouped into five clusters, as shown in the similarity index in the heat map. DP–25, RCMC–5, and Kasom Khullen in the cluster–I possessed lower incidence with low H_2O_2 and MDA content. Similarly, Cluster–V contains 13 genotypes with higher disease incidence.

3.3 AsA–GSH Pathways

The enzymatic and non–enzymatic antioxidants of AsA–GSH pathways showed a significant variation among the tested genotypes owing to *Pc* infection (Fig. 4A–F). AsA content recorded to be higher (1.33 mg g^{-1} FW) in RCMC–5 and lower in RCMC–4 and RCMC–9 (each of 0.70 mg g^{-1} FW) [Fig. 4A]. GSH was ranged between 2.53 (Jhankri)–7.33 (DP–25) µmol g^{-1} FW under epiphytotic *Pc* infection (Fig. 4B). Higher APX was observed in RCMC–5 (466.52 µM $min^{-1}g^{-1}$ FW), followed by Muktakeshi (369.64 µM $min^{-1}g^{-1}$ FW) and lower APX was recorded in RCMC–9 (136.16 µM $min^{-1}g^{-1}$ FW) [Fig. 4C]. MDAR content was ranged between 42.34–114.92 µM $min^{-1}g^{-1}$ FW whereas, DHAR was observed in the range of 186.16–533.63 µM $min^{-1}g^{-1}$ FW among the tested genotypes (Fig. 4D and E). Higher GR was observed in TSL (379.28 mM $min^{-1}g^{-1}$ FW) followed by DP–25 (372.35 mM $min^{-1}g^{-1}$ FW), Jhankri (360.38 mM $min^{-1}g^{-1}$ FW), RCMC–3 (356.60 mM $min^{-1}g^{-1}$ FW), RCMC–1 (351.56 mM $min^{-1}g^{-1}$ FW) and RCMC–5 (340.22 mM $min^{-1}g^{-1}$ FW) and lower GR was registered in RCMC–9 (214.21 mM $min^{-1}g^{-1}$ FW) [Fig. 4F].

The heat map (Fig. 5) depicted a significant difference in the antioxidants associated with AsA–GSH pathways among thirty genotypes of taro under *Pc* infection. The antioxidants were grouped into three clusters based on their regulations in taro genotypes against *Pc*. RCMC–5 and DP–25 grouped in cluster I, which possessed resistance with higher antioxidants. AsA showed a predominant role in association with APX and MDAR to scavenge the ROS. Based on the similarity index, the tested genotypes were grouped into three major clusters.

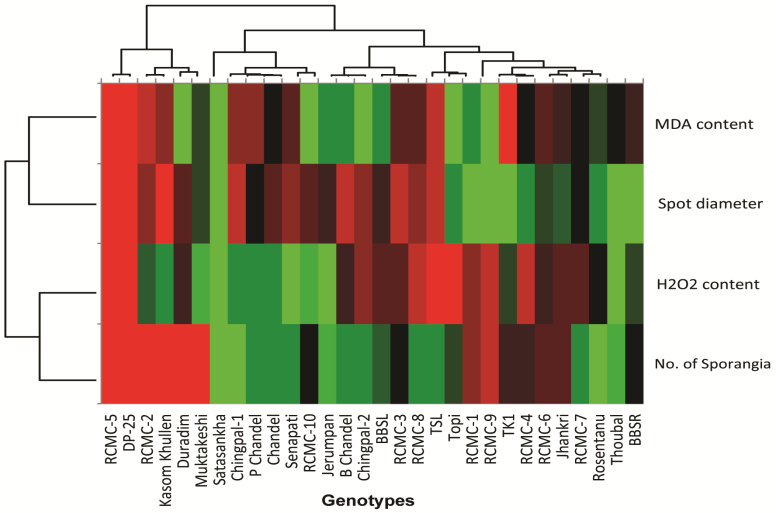

Fig. 3. Heat map depicting *Pc* incidence, H_2O_2 and MDA content in leaf tissues of thirty genotypes of taro owing to *Phytophthora colocasiae* infection

Color scale: Red (lower) to green (higher) through black; Color calibration: Automatic (Min:–1/Max:+1)

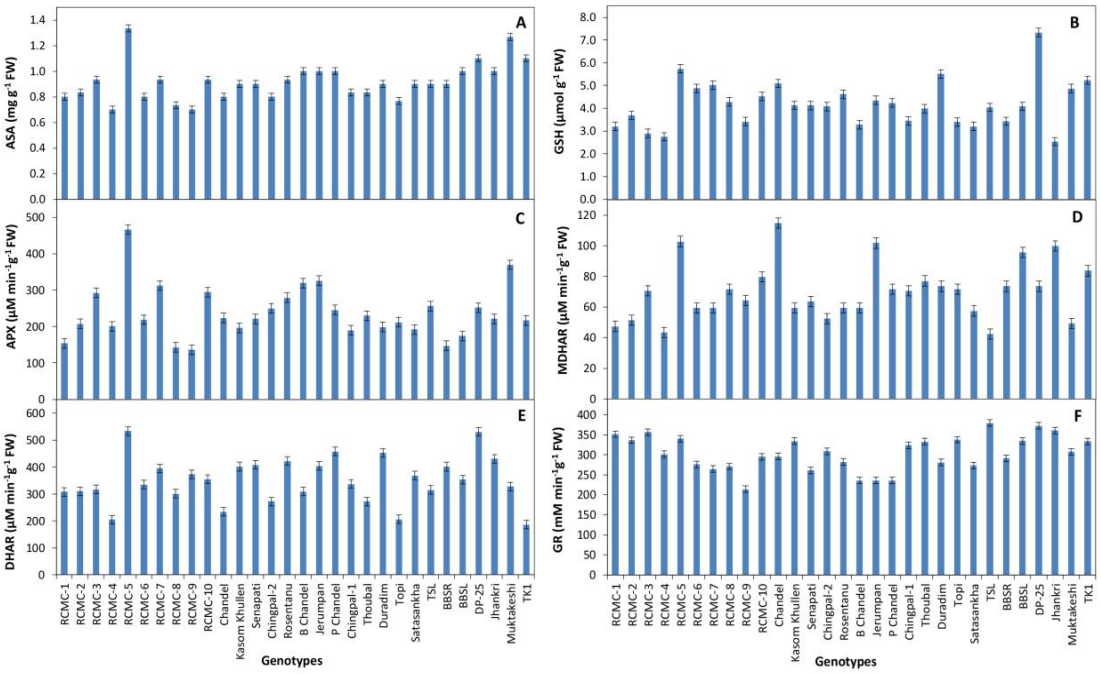

Fig. 4A–F. Differential occurrence of AsA–GSH cycle in thirty genotypes of taro owing to *Phytophthora colocasiae* infection. A: ascorbate (AsA, mg g^{-1} FW), B: reduced glutathione (GSH, µmol g^{-1} FW), C: ascorbate peroxidase (APX, µM $min^{-1}g^{-1}$ FW), D: monodehydro ascorbate reductase (MDAR, µM $min^{-1}g^{-1}$ FW), E: dehydro ascorbate reductase (DHAR, µM $min^{-1}g^{-1}$ FW), F: glutathione reductase (GR, mM $min^{-1}g^{-1}$ FW). Values are the mean of three replicates and bars represent standard error of means

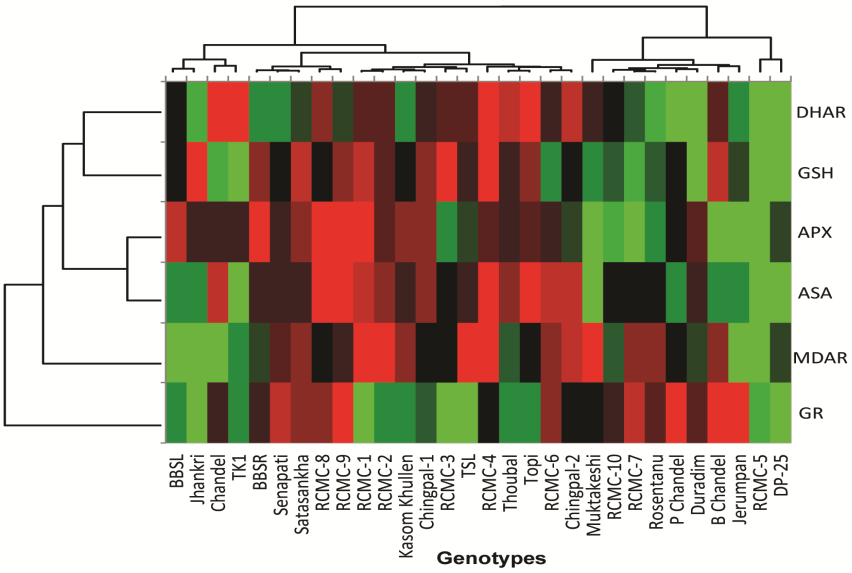

Fig. 5. Heat map depicting AsA–GSH pathways in thirty genotypes of taro owing to *Phytophthora colocasiae* infection

Color scale: Red (lower) to green (higher) through black; Color calibration: Automatic (Min:–1/Max:+1)

Plants evolved antioxidative machinery to scavenge ROS produced under stress [24]. Foyer and Noctor (2011) [25] opined that AsA and GSH are the heart of the redox mechanism in plant cell. AsA was reported to be one of the potent ROS scavenger and electron donor to APX to convert H_2O_2 to H_2O [25]. Gill and Tuteja (2010) [26] reported that the induction of APX is essential to remove the toxic effect of H_2O_2 at the cellular level. Higher APX induction is associated with *Phytophthora* blight in soybean [27]. Resistant genotypes express higher induction of antioxidants under stress than susceptible ones [6]. The oxidized ascorbate is generated by MDAR and dehydroascorbate is reduced to ascorbate by DHAR at the expenses of GSH [28]. GR acts to scavenge ROS in the plastid compartment of the plant cell [25].

In the present study, RCMC–5, among the tested taro genotypes, registered consistently higher induction of AsA, GSH, APX, MDAR, and DHAR to encounter the *Pc* incidence and overproduction of ROS. However, GR was more prominent in DP–25, Jhankri, and TSL. Similarly, Satasankha showed lower induction of AsA–GSH related antioxidants resulted in higher disease incidence. The content of antioxidants was more elevated in resistant genotypes than the susceptible ones. Komi et al. (2020) [29] reported that resistance or susceptibility depends upon the equilibrium between ROS and antioxidants. The result of this study evidenced that ROS's overproduction due to *Pc* infection was scavenged by AsA–GSH pathways to manage the leaf blight disease incidence in taro. The AsA–GSH cycle could be considered as a critical component while undertaking the taro–*Pc* resistance studies. It can also be used as a screening tool to assess the genetic diversities of taro for *Pc* resistance studies.

4. CONCLUSION

The present study concluded that AsA–GSH cycle regulates the ROS scavenging mechanism in taro–*Phytophthora* pathosystem. AsA–GSH associated antioxidants were induced highly in the resistant genotypes owing to *Pc* infection while compared with the susceptible genotypes. AsA–GSH encounters the ROS overproduction, which was confirmed with lower H_2O_2 and MDA content in resistant genotypes that resulted in lower *Pc* infection. AsA–GSH cycle could be a reliable parameter for selecting resistant lines while augmenting breeding strategies in taro against *Phytophthora colocasiae*.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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