MATERIALS AND METHODS
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2.1. Materials

Tomato seeds of varieties Pusa ruby and PKM - 1 were used. Jasmonic acid, Alpha TC and ascorbic acid were procured from Sigma-Aldrich, Co, St Louis MO (USA). Well-Bloom Agro Products (Mysore, India) consisting of 1% triacontanol was used as the source of TRIA. Plant growth promoters such as benzyl amino purine, alpha-naphthalene acetic acid, indole-3-acetic acid and indole-3-butyric acid (IBA), kinetin and chemicals such as thiobarbituric acid, nitro blue tetrazolium chloride, polyvinyl – pyrrolidone, Triton X-100, dithiothreitol and all vitamins were procured from HiMedia Laboratories (Mumbai, India). Other chemicals like potassium iodide, hydrogen peroxide and major as well as minor salts for Murashige and Skoog media (1962) were procured form Merck Specialities Private Limited (Mumbai, India) and Qualigens fine chemicals (Mumbai, India) and all were of analytical grade. D₂O used for spectral acquisition was of Spectroscopic grade.

2.2. Growth of seedling

2.2.1. Seed germination in pots

Seeds (Pusa ruby and PKM – 1) of the same size were methodically selected and germinated in pots containing 1:1:1 mixture of soil, fine sand and vermiculite and maintained in green house (Fig 10). Third leaves (Fig 11) from one month old tomato plant were taken as the source of explants.

2.2.2. In vitro seed germination

The seeds (Pusa ruby and PKM – 1) were washed thoroughly in running tap water, for 5 minutes followed by meticulous rinsing with distilled water to remove the dust particles on the surface. Under aseptic conditions seeds were surface sterilized with 70 % ethanol for one minute, which was followed by soaking them in 4 % sodium hypochlorite for 15
minutes and then they were rinsed with sterile distilled water for 5 - 6 times. Then seeds were transferred to tubes containing half strength MS media (Murashige and Skoog 1962) and kept in dark overnight, followed by incubation at 25 ± 2 °C under 16/8 h (dark/light) photoperiod with light intensity 40-50 μ mol/m²/s provided by cool white fluorescent lights.

2.3. Preparation of explants and in vitro culture conditions

In the variety Pusa ruby third leaf from one month old tomato plant was chosen as explants for callus induction. In the variety PKM-1, whole seeds, cotyledon and radical parts of the cut seeds, cotyledon as well as hypocotyledon from 10 days old seedlings and third leaves from one month old tomato plants were subjected to callus induction. In case of cotyledon and radical parts of the cut seed explants, seeds were initially sterilized with sodium hypochlorite and pre-cultured for 48 h on filter papers that were previously soaked in sterile distilled water. Then the seeds were cut into 2 pieces in such a way that one consisting of radical part and the other cotyledon part, along with whole seeds, were used as explants for callus induction. Leaves, cotyledons and hypocotyledons obtained from plants maintained in pots were washed thoroughly for 5 minutes in running tap water. Under aseptic conditions, they were surface sterilized with mercuric chloride (0.05%) for 5 minutes followed by 70% ethanol for 30 sec and then, rinsed thrice with sterile distilled water. Further, sterilized leaves as well as cotyledons and hypocotyledons were cut into small pieces and used as source of explants.

Leaf explants (5x5 mm), cotyledons as well as hypocotyledons (1 – 1.5 cm), whole seed, cotyledon and radical parts of the cut seeds were placed in bottles containing MS basal salt (Murashige and Skoog 1962), 3.0% sucrose (w/v), and with varying concentrations of benzyl amino purine (BAP), alpha-naphthalene acetic acid (NAA) and indole-3-acetic acid (IAA) (Table 1 and 2) for callus induction. The pH of the medium was set to 5.7 ± 0.2 and was solidified with 0.7% (w/v) agar. Culture bottles were maintained at 25 ± 2°C under 16/8 h (dark/light) photoperiod with light intensity 40-50 μ mol/m²/s provided by cool white fluorescent lights. Calluses (3 weeks old) obtained from leaf explants were
transferred to differentiating media which contained MS basal salts (Murashige and Skoog 1962), supplemented with different concentrations of plant growth regulators (PGR) such as benzyl amino purine (BAP), indole-3-acetic acid (IAA), alpha-naphthalene acetic acid (NAA) and kinetin for shoot regeneration [shoot regeneration media (SRM)] (Table 3). Different concentrations of IBA were used for root regeneration [root regeneration media (RRM)] (Table 4) and incubated in the same culture conditions as that of callus induction.

Effect of three different concentrations of each of TRIA, alpha TC and AA on shoot and root morphogenesis of cv. PKM – 1 (Table 6) and TRIA alone on shoot and root morphogenesis of cv. Pusa ruby (Table 5) variety were studied by placing the calluses in RRM/SRM supplemented with respective treatments. Medium without TRIA, alpha TC and AA served as the control. Periodic observations were carried out for the emergence and the numbers of roots/shoots.

2.4. Free radicals and antioxidant enzyme analysis

Effect of TRIA (T1- 1.14 µM, T2- 2.28 µM and T3- 4.56 µM), JA (T1- 0.001µM, T2- 0.01 µM and T3- 0.1 µM), TRIA+JA (2.28 µM TRIA + 0.001µM JA) and alpha TC (T1- 0.25 mM, T2- 0.5 mM and T3- 1 mM) on free radicals and antioxidant enzymes were assessed during root and shoot morphogenesis at periodic intervals. Calluses were transferred to MS basal salts (Murashige and Skoog 1962), supplemented with 12.3 µM benzyl amino purine (BAP) as well as 0.57 µM indole-3-acetic acid (IAA) and 4.9 µM indole-3-butyric acid (IBA) along with TRIA or JA or TRIA-JA or alpha TC, for shoot (SRM) and root (RRM) regeneration respectively. Analysis was performed with cultured tissues harvested from RRM as well SRM of Pusa ruby and from RRM of PKM-1 variety, for four continuous days at an interval of 24 h.
2.4.1. Measurement of hydrogen peroxide content

Hydrogen peroxide (H$_2$O$_2$) content was measured using the method followed by Sergiev et al., (1997). 500 mg of cultured tissue was homogenized with 5 ml of 0.1% (w/v) TCA and centrifuged. 0.5 ml of the supernatant was added to 0.5 ml of potassium phosphate buffer (10 mM) at pH 7.0 and 1 ml of KI (1 M). The mixture was incubated in dark at room temperature for 1 h. The absorbance of the supernatant was measured at 390 nm. The amount of H$_2$O$_2$ was determined using the extinction coefficient 0.28/µM/cm.

2.4.2. Determination of lipid peroxidation

Lipid peroxidation was assessed by means of malondialdehyde content (MDA) using Thiobarbituric acid (TBA) test followed by Heath and packer (1968). 500 mg of cultured tissue was homogenized in 5 ml of 0.1% (w/v) TCA solution and was subjected to centrifugation. Supernatant (0.5 ml) was added to 1 ml of 0.5% (w/v) TBA in 20% TCA. The mixture was incubated in boiling water for 30 min and centrifuged. The absorbance of the supernatant was read at 532 nm and corrected for interfering absorbance at 600 nm. MDA amount was determined using the extinction coefficient of 155/mM/cm.

2.4.3 Enzyme extraction

Samples of cultured tissue (500 mg) were homogenized in 5 ml of 100 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM ethylenediamine- tetraacetic acid (EDTA), 1 % (w/v) polyvinyl – pyrrolidone (PVP), 1 mM DTT and 0.5 % (v/v) Triton X-100 at 4$^\circ$ C. The homogenate was subjected to centrifugation at 18,000 g for 20 minutes at 4$^\circ$ C. The resultant supernatant was used to determine the activity of antioxidant enzymes (Lee and Lee 2000).
2.4.4. **SOD activity**

Superoxide dismutase (SOD) was assayed following the method of Superoxide dismutase activity by Beauchamp and Fridovich (1971) by measuring its capacity to inhibit photochemical reduction of nitro blue tetrazolium chloride (NBT). The reaction mixture (4 mL) contained 63 μM NBT, 13 mM methionine, 0·1 mM EDTA (ethylene diamine tetraacetic acid), 13 μM riboflavin, 0·05 M sodium carbonate and 0·5 mL enzyme extract (distilled [0·5 mL] water in the case of the control). Test-tubes were kept under 15 Watts fluorescent lamps for 20 min and then transferred to the dark for 20 min. The absorbance was determined at 560 nm and activity of the enzyme was expressed as units per mg of protein. One unit of the enzyme activity was defined as the enzyme required to inhibit (50 %) the reduction of NBT in comparison with the tubes lacking the enzyme.

2.4.5. **Catalase activity**

The activity of catalase was assayed by measuring the initial rate of disappearance of H₂O₂ by the method of Kato and Shimizu (1987) with minor modifications. Three ml of catalase assay reaction mixture contained 10 mM potassium phosphate buffer, pH 7.0, 5 μl of enzyme extract and 50 μl of 3% H₂O₂. The decrease in H₂O₂ was followed as decline in optical density at 240 nm, and the enzyme activity was estimated using the extinction coefficient (40 mM⁻¹cm⁻¹) for H₂O₂.

2.4.6. **Statistical analysis**

The values reported in the graphs are the means of three independent experiments with three replicates. Statistical dissimilarities between means (p ≤0.05) of control and treatments on each day were determined by two-way ANOVA followed by Tukey’s multiple range test.
2.5. Metabolomic analysis

Effect of TRIA (2.28 µM) JA (0.001 µM) and TRIA+ JA (2.28 µM TRIA + 0.001µM JA) on metabolic profiling during root morphogenesis was assessed. Calluses were transferred to RRM supplemented with TRIA, JA and TRIA+JA. Cultured tissue were harvested at 0, 3, 6, 9, 12, 24, 36, 48 as well as 72 h and subjected for NMR spectral analysis to comprehend the change in metabolic profile during early stages of root morphogenesis.

2.5.1. Metabolite extraction

750 mg of frozen cultured tissue was ground to a fine powder with liquid nitrogen using a mortar and pestle. Homogenate was extracted in three 8 ml aliquots of 80 % (v/v) ethanol. Pooled aliquots were centrifuged for 10 minutes. The supernatant was lyophilized in a vacuum centrifuge at 50˚C, re-dissolved in 5 ml distilled water and re-lyophilized. Samples were re-dissolved in D₂O at pH 7 and formate was used as internal standard.

2.5.2. NMR spectroscopy

2.5.2.1. Spectral acquisition

All 1D ¹H NMR spectra were acquired on Bruker AV-III spectrometer operating at 400 MHz proton resonance frequency equipped with 5mm BBI probe. A total of 640 transients were collected with 6730 complex data points ranging a spectral width of 4807.69 Hz at 298 K. Fourier transformation, phase correction and baseline corrections were applied. A line broadening of 0.3 Hz. was taken. Hetero nuclear Single Quantum Coherence spectroscopy (HSQC) was performed to support chemical shift assignments.
2.5.2.2. Data analysis

Identification and quantification of each metabolite was executed using the profiler module of the Chenomx NMR suite v. 8.1 trial version (Chenomx. Inc., Edmonton, Canada). $^1$H NMR spectra were studied using a library containing 198 compounds, constructed by chemically designing compounds of interest using their peak center and $J$-coupling information. This library consists of the unique $^1$H NMR spectra of each standard compound documented at 600 MHz quantified by the addition of a known amount of internal standard (formate). Comparison of the NMR spectra with the standard metabolites in the library, provide a series of metabolites and their respective concentrations. After excluding all shifts associated to the solvent (i.e. in the range of 4.5– 5.0 ppm) and DSS, left behind spectral regions were divided into 0.04-ppm bins. The bins were normalized to the area under the formate peak to evaluate the contribution of individual metabolites to the spectrum as well as total spectral area to correct for dilution effects (Zulak et al., 2008).

2.5.2.3. Statistical analysis

Statistical analysis was performed with an aid of Excel – 2016 using unsupervised principal component analysis (PCA) and supervised partial least square enhanced discriminant analysis (PLS-EDA) (Trygg et al., 2007). PCA is a popular multivariate technique that is primarily used to reduce the dimensionality of multi-attributes to two or three dimensions (Huang et al., 2005). It summarizes the variation in correlated multi-attribute to a set of uncorrelated components. Each of them is a particular linear combination of the original variables. Thus extracted uncorrelated components are called principal components. First few PC’s include most of the variations in the original dataset. First principal component accounts for the co-variance that is shared by all attributes; this may be a better estimate than simple or weighted averages of the original variables. PLS-EDA is a supervised analytical tool that was used on specific time intervals to reveal differences in the metabolite profiles that are masked by PCA using all data points (Trygg et al., 2007). PLS-EDA focuses on variance due to treatments alone.
while minimizing other biological or analytical variables. For both PCA and PLS-EDA spectral regions were the X-matrix. For PLS-EDA, class difference (e.g. control versus treatment) was the Y-matrix. The eminence of each model was determined by the goodness of fit parameter (R2) and the goodness of prediction parameter depending on the fraction correctly predicted in a 1/7 cross-validation (Q2).