Material and Methods
Plant material and standardization of adventitious rooting response in sunflower hypocotyl explants

Seeds of sunflower (*Helianthus annuus* L. cv. Morden) were germinated on moist germination sheets at 25 ± 2°C under continuous illumination of 4.3 Wm².

The following variables were tried in the preliminary experiments:

i. *Age of explants*– light-grown 4, 5, 6 and 7 d old seedlings were chosen for the initial experiments.

ii. *Auxin concentrations* – Explants were subjected to 0, 5, 10 and 15 µM of IAA in water.

iii. *Types of explants* – With meristem intact (M); with both meristem and cotyledon intact (B), and both meristem and cotyledon removed (N).

For preparing explants, hypocotyls were cut at a position 6 cm away from the meristem, and according to the type of explants, the cotyledons were removed (M), kept intact (B) or both cotyledon and meristem were removed (N). These explants were incubated in glass corning vials with 1 mL of IAA (5, 10 or 15 µM) for 7 days and the rooting response was observed.

Pharmacological treatments

Hypocotyl explants with apical meristem intact but cotyledons excised (M) were selected for the present work with a view to provide a continuity of the endogenous auxin source. Similar explants were also recently used by Huang et al. (2007) to investigate indole-3-butyric acid (IBA)-induced AR formation in mung bean (*Phaseolus radiatus* L.). Using IAA instead of IBA for such investigations was preferred in the present work keeping in view that the two auxins employ different transport proteins for their polar transport (Rashotte et al., 2003). Freshly harvested explants were put upright in glass vials with their proximal cut ends dipped in 1 mL of different concentrations (5, 10 and 15 µM) of IAA, thus bathing the hypocotyl segments up to 6 mm of their lower ends. Explants were maintained in dark during the course of experiments. The number of AR visible on hypocotyl surface was recorded daily up to
7 days of incubation. Various concentrations of other test solutions, namely 1-naphthylphthalamic acid (NPA; auxin efflux blocker; 1 and 10 µM), Latrunculin B (Lat B; an inducer of actin depolymerization; 25, 50 and 100 nM), Cyclosporin A (CsA; an inhibitor of cyclophilins; 1, 5 and 10 µM), sodium nitroprusside (SNP; NO donor; 1, 5, 10 and 100 µM) and 2-phenyl-4,4,5,5-tetramethyllumidazoline-1-oxyl-3-oxide (PTIO; NO scavenger; 1 and 1.5 mM), were initially used to select their respective optimal concentrations. Based on these preliminary experiments NPA, Lat B, CsA, SNP and PTIO were used at 10 µM, 100 nM, 10 µM, 100 µM and 1.5 mM, respectively, for all subsequent experiments. Some other treatment combinations, namely NPA (10 µM) + IAA (10 µM); LatB (100 nM) + IAA (10 µM); CsA (10 µM) + IAA (10 µM); SNP (100 µM) + NPA (10 µM) and PTIO (1.5 mM) + IAA (10 µM) were also used to investigate their effects on adventitious rooting. Hypocotyl explants incubated in distilled water served as control. Morphological observations of rooting response were photographed after 7 days of incubation, using Nikon digital camera fitted on a stereomicroscope (Zeiss, Germany).

Clearing of hypocotyl explants for visualization of root initiation

Detailed evaluation of root initiation within the hypocotyl tissue was observed after clearing by immersing the explants in a 3:1 solution of ethanol: acetic acid overnight. They were then transferred to 2N NaOH solution, left overnight, washed once with distilled water and stained with safranin solution for 2-3 min. Excess stain was removed by repeated washing in distilled water. The lower 2 cm region of hypocotyl explants was then cut and mounted on a glass slide to examine endogenous root initials. Morphological rooting response was then photographed using a stereomicroscope (Zeiss, Germany) fitted with a Nikon camera.

Anatomical changes and epifluorescence microscopic visualization of fluorescence due to endogenous NO distribution using DAF-2DA

Hypocotyl explants subjected to various pharmacological agents (for a period of 4 d) were used for obtaining fine sections from the basal 6 mm regions and they were observed for various stages of AR formation, using a photomicroscope. Digital images
were taken using AxioCam digital camera (Zeiss, Germany) fitted on the microscope. Cross sections obtained from the basal 6 mm regions of various explants were incubated for 40 min in 10 µM of DAF-2DA prepared in 10 mM Tris-HCl buffer set at pH 7.4 (according to Corpas et al., 2006). Sections were then washed twice for 5 min each with the same buffer (without DAF-2DA) and observed for fluorescence due to nitric oxide (ex 485 nm; em 515 nm), using epifluorescence microscope (Axioskop from Zeiss, Germany). In order to check the specificity of fluorescence due to NO (control), sections were preincubated for 1 h with 1 mM PTIO in 10 mM Tris-HCl buffer (pH 7.4), subsequently incubated for 40 min with 10 µM DAF-2DA in presence of 1 mM PTIO and then examined for fluorescence quenching microscopically.

**Localization of actin**

Actin was visualized according to Blancaflor (2000), using Alexa Fluor phalloidin (Molecular Probes Inc., U.S.A) as the fluorescent probe. Fine sections from the basal 6 mm regions of explants treated with Lat B, IAA, NPA and PTIO till 4 days, were immersed for 30 min in 50 mM PIPES buffer (pH 6.9) containing 4 mM MgSO$_4$ and 10 mM EGTA. Sections were then incubated for 10 min in the same buffer containing 0.1 µM Alexa Fluor phalloidin, 0.3 M mannitol and 2% glycerol. Sections, subsequently washed once with PIPES buffer, were mounted on a glass slide and immediately observed for fluorescence under CLSM using argon laser.

**Estimation of NO production due to putative Nitric Oxide Synthase (NOS) activity**

Putative NOS activity was estimated using FCANOS-kit (Sigma-Aldrich, USA), according to Annie-Jeyachristy et al. (2008). Briefly, 1 g tissue from the basal 6 mm hypocotyl segments (pooled from several explants subjected to specified treatments till 4 d) was homogenized in the extraction buffer (50 mM Tris-HCl, 250 mM sucrose and 1 mM EDTA, pH-7.4) containing 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 2 µg mL$^{-1}$ aprotinin, 25 µg mL$^{-1}$ soybean trypsin inhibitor and 10 µL g$^{-1}$ protein inhibitor cocktails procured from Sigma (USA). The homogenate was frozen and thawed with liquid nitrogen thrice and centrifuged at 10,000 g for 20 min at 4°C, according to Butt et al. (2003). The supernatant was
collected and stored at -20°C. Protein concentration was determined by Bradford assay (Bradford, 1976). Total NO content was estimated in triplicate for each sample in a reaction buffer containing 1 mM L-arginine and 1 µM β-NADPH. After addition of 10 µM DAF-2DA, the reaction mixture was incubated at 25°C for 30 min, according to Corpas et al. (2004). In order to determine putative NOS activity, samples were preincubated with 10 mM aminoguanidine (inhibitor for the activity of inducible NOS) in the reaction buffer for 30 min, followed by the addition of 1 mM L-arginine, 1 µM β-NADPH and 10 µM DAF-2DA (Valderrama et al., 2007). The reaction mixture was incubated at 25°C for 30 min. Reaction mixture without protein served as control for the respective sample. Fluorescence was measured using a spectrofluorometer (Model: FS920; Edinburgh Instruments, UK) at the excitation and emission wavelengths of 485 and 515 nm, respectively, according to Kojima et al. (1998). Data was presented as fluorescence units (counts per second).

**Synthesis and characterization of MNIP-Cu: a specific fluorescent probe for detection of nitric oxide**

(4-methoxy-2-(1H-naphtho[2,3-d]imidazol-2-yl)phenol) [MNIP] was synthesized according to Ouyang et al. (2008) with minor modifications. A mixture of 2-hydroxy-4-methoxybenzaldehyde (0.316 mmol), 2, 3-diaminonaphthalene [1] (0.316 mmol) and nitrobenzene [2] (2 mL) was refluxed for 2 h. After completion of reaction, the reaction mixture was cooled down to room temperature and diluted with hexane. The precipitate of MNIP [3] formed was filtered and washed with diethyl ether.
MNIP was characterised by $^1$H NMR (JEOL Delta Spectrometer, Japan) and IR spectroscopy (2000 FT-IR, Perkin-Elmer). NMR spectra were recorded in $d_6$-DMSO, using tetramethyl silane (TMS) as internal standard.

In order to achieve the synthesis of MNIP-Cu, crystals of MNIP were dissolved in dimethyl sulfoxide (DMSO) to obtain a 10 mM stock, which was stored at -20°C. MNIP-Cu was always prepared fresh from MNIP just before use. MNIP stock (10 mM) was diluted to 1 mM with DMSO and 20 µL of 50 mM of aqueous copper sulphate was added to 1 mL of MNIP solution. The mixture was stirred for 5 min at room temperature, resulting in the formation of a stable yellow coloured solution of MNIP-Cu.

Detection of NO in hypocotyl explants

Hypocotyl explants at different stages of adventitious rooting from their basal ends in response to 10 µM IAA treatment, were treated with 50 µM MNIP-Cu and visualized for fluorescence due to NO within 15 min. Visualization of NO fluorescence due to MNIP-NO complex was achieved using UVP EC3 imaging system (ex 385 nm ; em 420 nm) and imaged using the attached camera.

NO production in hypocotyl-derived protoplasts in response to auxin treatment

Protoplasts were isolated from the hypocotyl of 4 d old, light-grown seedlings. Purified protoplast preparations were treated with 10 µM IAA, 10 µM NPA or 100 µM SNP for 30 min, followed by treatment with 25 µM of MNIP-Cu. Protoplasts were visualized for fluorescence due to NO after exciting at 365 nm (em. 420 nm). For each of the above treatments, incubation with 1 mM PTIO (NO scavenger) for 30 min, followed by co-incubation with MNIP-Cu for 10 min, was also undertaken. All observations were taken on a fluorescence photomicroscope at 630X.

Hypocotyl protein profile by one dimensional SDS-PAGE

One gram tissue from the basal 6 mm hypocotyl segments [pooled from several explants subjected to specified treatments (IAA, NPA, Lat B, Cyclosporin) till 7 d, was homogenized in grinding medium (0.1 M Tris-NaOH, 0.4 M sucrose, 10 mM
KCl, 1 mM MgCl₂, 1 mM EDTA, 1 mM PMSF pH-7.5 and 0.2% mercaptoethanol) in a proportion of 3 mL g⁻¹ FW. The homogenates were filtered through 4 layers of muslin cloth and centrifuged at 10,000 g for 20 min at 4°C. TSP collected as supernatant was acetone-precipitated overnight. Subsequently, the pellet was resolublized in grinding medium, quantified by Bradford method for protein estimation and reconstituted in Lamelli buffer. 60 µg protein was loaded for single dimension separation by SDS-PAGE. The gel was run at 22°C at constant current of 25 mA. The difference in band intensities of IAA, NPA, Lat B and CsA was plotted using Quantity one software (Biorad, USA).

*i. Stock solutions for SDS-PAGE analysis (According to Laemmli, 1970):* Acrylamide stock (30%) was prepared by dissolving 29.2 g of acrylamide and 0.8 g bisacrylamide in water to make a final volume of 100 mL.

- Resolving gel buffer (3 M Tris buffer, pH 8.8) was prepared by dissolving 36.35 g of Tris base in water. The pH of solution was set at 8.8 with HCl and final volume was made to 100 mL using distilled water.
- Stacking gel buffer (0.5 M Tris buffer, pH 6.8) was prepared by dissolving 6.06 g of Tris base in water. The pH of solution was set at 6.8 with HCl and final volume was made to 100 mL using distilled water.
- Sodium dodecyl sulphate (SDS) solution (10%; w/v) was prepared by dissolving 10 g of SDS in water to make a final volume of 100 mL.
- Ammonium persulphate (APS) solution (10%; w/v) was prepared by dissolving 100 mg APS in water to make a final volume of 1 mL.
- TEMED (N,N,N’,N’-tetramethylethylenediamine) was used undiluted.
- Electrode buffer [0.025 M Tris, 0.192 M glycine, 0.1% (w/v) SDS] was prepared by dissolving 15.15 g Tris base, 72 g glycine and 5 g SDS in water to make a final volume of 5 L.

The acrylamide stock, resolving gel buffer, stacking gel buffer and TEMED were stored at 4°C. Ammonium persulphate solution was freshly prepared. SDS solution was stored at room temperature.
**ii. Preparation of resolving linear gradient SDS gel:** Resolving linear gradient gels (10 to 20%) were prepared by mixing the resolving gel mixtures (10% and 20%), respectively, using a gradient mixer. The composition of resolving gel mixtures is shown in the following table.

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentrations of resolving gel mixture</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>10%</td>
</tr>
<tr>
<td>Acrylamide stock (40% T)</td>
<td>3.8 mL</td>
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<tr>
<td>Resolving gel buffer (3 M Tris, pH 8.8)</td>
<td>1.9 mL</td>
</tr>
<tr>
<td>SDS solution (10%; w/v)</td>
<td>0.15 mL</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µL</td>
</tr>
<tr>
<td>Ammonium persulphate a (10%; w/v)</td>
<td>0.1 mL</td>
</tr>
<tr>
<td>Water b</td>
<td>as required</td>
</tr>
</tbody>
</table>

* a Freshly prepared and added at last to start polymerization
* b As required to bring the final volume to 15 mL

**iii. Preparation of stacking gel:** Stacking gel was prepared after polymerization of the resolving gel. Composition of stacking gel is as follows.

<table>
<thead>
<tr>
<th>Components</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide stock (40% T)</td>
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</tr>
<tr>
<td>Stacking gel buffer (0.5 M Tris, pH 6.8)</td>
<td>1.9 mL</td>
</tr>
<tr>
<td>SDS solution (10%; w/v)</td>
<td>0.15 mL</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µL</td>
</tr>
<tr>
<td>Ammonium persulphate a (10%; w/v)</td>
<td>0.1 mL</td>
</tr>
<tr>
<td>Water b</td>
<td>as required</td>
</tr>
</tbody>
</table>

* a Freshly prepared and added to start polymerization
* b As required to bring the final volume to 15 mL
iv. Laemmli sample buffer solution: The laemmli sample buffer solution [0.06 M Tris, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.008% (w/v) Bromophenol blue] was prepared by mixing 1.2 mL of stacking gel buffer (0.5 M Tris, pH 6.8), 1 mL glycerol, 2 mL of 10% (w/v) SDS stock, 0.2 mL of Bromophenol blue (0.4%, w/v) and 5.6 mL water to make final volume of 10 mL.

Protein samples mixed with two volumes of reducing Laemmli sample buffer and mercaptoethanol (10.52 µL ME per 200 µL Laemmli sample buffer) were heated in boiling water for 2 min for subsequent analysis by SDS-PAGE.

v. Staining and destaining of gels: SDS gels were stained with 0.2% (w/v) Coomassie blue R-250 dissolved in a mixture of methanol, acetic acid and water (4: 1: 5). Blue coloured bands, representing the resolved proteins, were visualized after destaining with the same solvent (mixture of methanol, acetic acid and water in 4:1:5).

Visualization of the distribution pattern of tyrosine-nitrated proteins in wax sections of hypocotyls using antibodies labelled with fluorescent tags

i. Selection of tissue for fixation: Lower portions (6 mm) of hypocotyl explants incubated in 1 mL of various pharmacological agents for 7 days were excised for fixation.

ii. Fixation, embedding and sectioning: Fixation of tissue samples and other subsequent steps were performed according to Amenós et al. (2009). Briefly, the excised tissue was fixed for 60 min in 3.7% formaldehyde prepared in stabilizing buffer (50 mM PIPES, 5 mM MgSO₄·7H₂O, 5 mM EGTA, pH 6.9) for 60 min The fixed sample was washed with stabilizing buffer for 30 min and subsequently with phosphate buffer saline [PBS, 0.14 M NaCl, 2.7 mM KCl, 6.5 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.3] for 15 min. The samples were then dehydrated at 4°C for 30 min each, in an increasing gradation of ethanol (30, 50, 70 and 90%, diluted with PBS). In the final step of dehydration, the samples were transferred into 97% ethanol for 30 min in hot water bath maintained at 37°C. An incubation of 10 min in 0.01% toluidine blue (prepared in 97% ethanol) was performed prior to embedding. Samples were
then left in 97% ethanol for 10 min at 37°C. The embedding medium consisted of low melting point Steedman’s wax (Baluška et al., 1992). It was prepared by melting PEG 400 distearate at 65°C and then mixing 1-hexadecanol (9:1, w/w) with continuous stirring for 3-4 h. Wax was then cooled at room temperature and stored. Embedding was initially followed in ethanol/wax (1:1) overnight. Finally, the samples were transferred to 100% wax for 6-7 days. Samples were then placed in the embedding moulds and left overnight for polymerization of wax. After embedding, wax ribbons of 10 µm thickness were placed on the slides coated with 0.1% polyethylenimine, and were evenly stretched using a drop of water. The slides were left overnight at room temperature.

iii. Immunofluorescence localization of nitrotyrosine sites by Confocal Laser Scanning Microscopy: Prior to immunolabelling, slides were dewaxed thrice in 97% ethanol, for 10 min each at 4°C. After a 10 min wash in pure ethanol, sections were rehydrated by passing through an ethanol gradation series (90%, 50% ethanol in PBS for 10 min each) and finally PBS (10 mins) at 4°C. Before the addition of primary antibody, sections were incubated in a blocking solution (2% BSA in PBS) and then washed in PBS for 5 min. Immunolabelling was done according to Valderrama et al. (2007). Briefly, the sections were incubated with a rabbit polyclonal antibody against 3-nitrotyrosine diluted to 1:300 in PBS containing 0.1% BSA (w/v) for 2 days at 4°C. Subsequently, sections were washed in PBS for 10 min and then incubated with Cy3-labelled anti-rabbit IgG (Amersham) diluted to 1:1000 in PBS containing 0.1% BSA (w/v) for 1 hr at room temperature. After labelling, a 10 min rinsing was done in PBS followed by treatment with 0.01% toluidine blue in PBS for 10 mins (Brown et al., 1989). After washing in PBS for 10 min, the slides were mounted using p-phenylenediamine-an anti-fade mounting medium (Krenik et al., 1989). The sections were visualized using confocal laser scanning microscope (Olympus FluoView, Germany) at excitation 550 nm and emission was monitored at 570 nm.
Sample preparation for detection of tyrosine-nitrated proteins from hypocotyl segments

One gram tissue from the basal 6 mm hypocotyl segments (pooled from several explants subjected to specified treatments (IAA, NPA, SNP, SNP+NPA, PTIO, PTIO+IAA) till 7 d of incubation, was homogenized in the grinding medium (0.1 M Tris-NaOH, 0.4 M sucrose, 10 mM KCl, 1 mM MgCl$_2$, 1 mM EDTA, 1 mM PMSF pH-7.5 and 0.2% mercaptoethanol) in a proportion of 3 mL g$^{-1}$ FW. The homogenates were filtered through 4 layers of muslin cloth and centrifuged at 10,000 g for 20 min at 4˚C (Total soluble protein; TSP). The TSP collected as supernatant was acetone-precipitated overnight. Subsequently, the pellet was resolublized in grinding medium, quantified by Bradford method of protein estimation and constituted in Lamelli buffer. 30 µg protein was loaded for single dimension separation in SDS-PAGE in Miniprotean Tetra Cell (Biorad, U.S.A.). The gel was run at 4˚C at constant voltage of 75V.

Western blotting for detection of tyrosine-nitrated proteins

Western blotting for tyrosine-nitrated proteins was done according to Butterfield and Sultana (2008). Briefly, after running the SDS-PAGE, the gel was sliced out and washed in transfer buffer [20% glycine, 5% tris(hydroxymethyl)aminomethane and 10% methanol] at 4˚C for at least 15 mins. Meanwhile, PVDF membrane was washed in methanol (100%) for 10 sec, followed by deionized water (5 mins) and then in transfer buffer (10-15 mins). Filter paper cut to the size of PVDF membrane, was pre-soaked in transfer buffer. The transfer sandwich was prepared by packing together filterpaper (2 pcs), activated PVDF membrane, gel, filterpaper (2 pcs) and placed on the semidry transfer unit platform (ECL semi-dry Blotter, Amersham Biosciences). A current of 0.8 mA per cm$^2$ of gel was provided for a period of 4 hrs at room temperature to achieve complete transfer of proteins. After the completion of transfer, the membrane was removed and stained with Ponceau S (a reversible protein binding stain, prepared by dissolving 0.5% Ponceau S in 1% acetic acid) to monitor proper transfer of proteins. Subsequently the blot was rinsed with MilliQ water to completely remove the stain and then incubated in blocking buffer [2% BSA, 0.01% sodium
azide, 0.2% Tween 20 in phosphate buffer saline (PBS, 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄, final volume=1L) pH 6.8] overnight at 4°C. Blocking prevents non-specific binding of primary and secondary antibodies to membrane. The membrane was then incubated with anti-3NT antibody (obtained from Sigma-Aldrich, USA) in a dilution of 1:1000 in blocking buffer, for 2 h at room temperature on an orbital shaker. Thereafter, the membrane was washed in wash buffer (0.01% sodium azide, 0.2% tween 20 in PBS pH 6.8) three times for 5 mins each and incubated in secondary antibody (anti-rabbit IgG conjugated to alkaline phosphatase antibody) (1:3000 in wash buffer) for 1 h at RT on an orbital shaker. Finally, the membrane was washed in wash buffer three times for 5 mins each and developed using freshly prepared BCIP/NBT (1 Sigma Fast tablet dissolved in 10 mL milliQ water) for 10-30 min. BCIP/NBT (5-Bromo-4-chloro-3-indoyl phosphate/Nitro blue tetrazolium) is used as a precipitating substrate for detection of alkaline phosphatase activity. Once the desirable colour intensity was obtained, the membrane was placed in MilliQ water. Membrane dried between tissue paper could be stored. Both primary and secondary antibodies were obtained from Sigma-Aldrich (USA).

Detection of Superoxide dismutase (SOD; EC 1.15.1.1) activity during adventitious root formation

i. Zymographic detection of Superoxide dismutase activity in basal portion of hypocotyl segments: Superoxide dismutase (SOD) activity was estimated according to Shine et al. (2012), with minor modifications. Briefly, 1 g tissue from the basal 6 mm hypocotyl segments (pooled from several explants subjected to specified treatments of IAA, NPA and SNP incubated for 7 days, was homogenized in 50 mM tris-HCl buffer, pH 4 (containing 50 mM NaCl, 0.05% Tween 80, 1 mM PMSF) in a proportion of 3 mL g⁻¹ FW. The homogenates were centrifuged at 10,000 g for 20 min at 4°C. Supernatant was collected and protein quantification was done by Bradford method. 50 µg protein was constituted in native Lamelli buffer (Lamelli buffer without SDS) and loaded for single dimension separation in 10-20% SDS-PAGE at 4°C (conditions: 75 V for 0.5 h, 25 mA for 5 h) using a vertical gel electrophoresis unit (LKB, Sweden). Gel was then incubated in 2.5 mM Nitroblue tetrazolium (NBT) for 25 min in dark and thereafter transferred to 50 mM phosphate buffer (pH 7.8),
containing 28 µM riboflavin and 28 mM TEMED, for 20 mins in dark. The gel was subsequently rinsed and observed on a light box for 10-15 min till clear bands showing SOD activity appeared on a blue background.

**ii. Spectrophotometric estimation of SOD activity:** SOD activity was estimated spectrophotometrically, according to Beauchamp and Fridovich (1971), with minor modifications. Sample preparation was done similar to that for zymographic detection of SOD. Subsequently, 50 µg protein was added to 3 mL reaction mixture (containing 1.67x10^-4 M NBT, 9.9 M methionine, 0.025% Triton X100 and 2.4 µM riboflavin dissolved in 50 mM sodium phosphate buffer, pH 7.8), and incubated in front of 300 Watt light source for 10 min to perform the photo-induced reaction. Protein incubated with reaction mixture in dark served as blank and the reaction buffer devoid of enzyme, incubated in light served as control. Readings were taken in triplicate at 560 nm. Enzyme activity from spectrophotometric analysis was expressed as units of SOD activity per mg of protein.

**Peroxidase (POD; EC 1.11.1.7) activity during adventitious root formation**

**i. Zymographic detection of peroxidase activity in hypocotyl explants subjected to various pharmacological treatments:** Peroxidase activity was estimated according to Alba et al. (1998), with minor modifications. Briefly, 1 g tissue from the basal 6 mm hypocotyl segments (pooled from several explants subjected to specified treatments IAA, NPA, SNP) incubated for 7 days, was homogenized in 50 mM sodium acetate buffer, pH 4, in a proportion of 3 mL g^-1 FW. The homogenates were centrifuged at 10,000 g for 20 min at 4°C. Supernatants collected were acetone-precipitated overnight. Subsequently, the pellets were air-dried and resolublized in Tris-HCl buffer (pH 7.5). Protein was quantified by Bradford method and constituted in native Lamelli buffer (Lamelli buffer without SDS). 20 µg protein was loaded for single dimension separation in 10-20% SDS-PAGE at 4°C (conditions: 75 V for 0.5 h, 25 mA for 5 h) using a vertical gel electrophoresis unit (LKB, Sweden). Gel was then incubated in 0.2 M sodium acetate buffer (pH 5) containing 1.3 mM benzidine and 1.3 mM H₂O₂ till brown bands developed.
ii. Spectrophotometric estimation of peroxidase activity: 10 µg protein (resuspended in Tris-HCl buffer, pH 7.5) was added to 2.4 mL substrate solution (0.6 mM o-dianisidine, 8.8 mM H₂O₂ in 50 mM sodium phosphate buffer, pH-6) and the absorbance value was recorded at 460 nm for 10 min at an interval of 1 min. Substrate buffer containing Tris-HCl buffer served as blank. Enzyme activity from spectrophotometric analysis was expressed as specific activity of POD in Mol µg⁻¹ min⁻¹).

Two dimensional polyacrylamide gel electrophoresis of total soluble protein from hypocotyls

i. Protein extraction from hypocotyl segments: One gram of hypocotyl segments (basal 6 mm region) from various pharmacological treatments (IAA, SNP, NPA, PTIO, SNP+NPA and PTIO+IAA) were homogenized in 5 mL of phosphate saline buffer (pH 7.6) containing 65 mM K₂HPO₄, 2.6 mM KH₂PO₄, 400 mM NaCl and 3 mM NaN₃ at 4ºC, using a glass mortar and pestle on ice. The homogenate was centrifuged at 15,000 g for 10 min. Trichloroacetic acid (TCA) was added to the supernatant to a final concentration of 10%. The solution was kept on ice for 30 min and then centrifuged for 10 min at 15,000 g at 4ºC. The resultant precipitate was homogenized with 300 µL of lysis buffer (8.5 M urea, 2.5 M thiourea, 5 % CHAPS, 100 mM DTT; 0.5 % IPG buffer) using a glass mortar and pestle on ice. The homogenate was centrifuged twice at 15,000 g at room temperature for 10 min. The supernatant was subjected to electrophoresis. Contaminating salts were removed by dialysis using the Quant dialysis kit (GE Healthcare). To determine the protein concentration, 2D-Quant-kit (GE Healthcare) was used according to the manufacturer’s instructions.

ii. 2-D gel electrophoresis: The first electrophoretic dimension (isoelectric focusing) was carried out on an IPGphor II unit (GE Healthcare) by loading 200 µL of sample (200 µg protein) onto a 13 cm immobilized pH gradient (IPG) strip (GE Healthcare, pH 4-7). After 14 h of rehydration, the proteins were focused by application of a voltage gradient (250 V for 1 h, 500 V for 1 h, 4000 V for 1 h and 4000 V for 5:35 h). Subsequent to isoelectric focusing, the strips were equilibrated for 15 min in
equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerine, 2% (w/v) sodium dodecyl sulfate (SDS), 20 mM DTT, 0.01% bromphenol blue).

The second electrophoretic dimension was performed on a 12.5% SDS-polyacrylamide gel (conditions: 75 V for 0.5 h, 150 V for 5 h) using a vertical gel electrophoresis unit (LKB, Sweden). Proteins were visualized with colloidal Coomassie brilliant blue G-250.

**iii. Preparation of Coomassie brilliant blue G 250 staining solution**

Solution A: It was prepared by dissolving 16 mL of orthophosphoric acid in 768 mL of distilled water. Eighty grams of ammonium sulphate was subsequently dissolved in this solution.

Solution B: Prepared by dissolving 1 g Coomassie brilliant blue G 250 in 20 mL distilled water following by stirring.

Solution C: Prepared by mixing 16 mL of solution B into solution A so that the final volume was 800 mL.

Solution D: Prepared by adding 200 mL of methanol to solution C, while stirring just before use.

The gels were analyzed by PD Quest software from Bio-Rad (USA).

**Immunohistochemical localization of serotonin in hypocotyl explants subjected to pharmacological treatments**

i. **Selection of tissue for fixation:** Lower portions (6mm) of hypocotyl explants incubated in 1 mL of various pharmacological agents for 7 days were excised for fixation.

ii. **Fixation, embedding and sectioning:** Fixation of tissue samples and other subsequent steps were performed according to Kang et al. (2009). Briefly, the excised tissue was fixed for 60 min in 0.05% glutaraldehyde and 4% p-formaldehyde prepared in phosphate buffer saline [PBS, 0.14 M NaCl, 2.7 mM KCl, 6.5 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.3]. The samples were then dehydrated in an increasing gradation
of ethanol (70, 80 and 90%, diluted with PBS) for 3 h each. In the final step of
dehydration, the samples were transferred twice into 100% ethanol for 3 h each. Prior
to wax infiltration, the samples were transferred through an increasing gradation of
xylene (50, 75 and 100% for 6, 3 and 6 h, respectively) in ethanol. The samples were
then cold infiltrated in wax saturated xylene for 24 h at room temperature.
Subsequently, samples were infiltrated with fresh wax at 40˚C, 50˚C and 60˚C for 24
h each. Finally, the samples were transferred into fresh wax and subjected to extended
infiltration at 60˚C for 4 days. Samples were then placed in the embedding moulds
containing molten wax and left overnight for polymerization. After embedding, wax
ribbons of 8 µm thickness (prepared by microtome) were placed on slides coated with
0.1% polyethylenimine, and were evenly stretched using a drop of 4% formaldehyde.
The slides were then placed on a hot plate (35-40˚C) for 15-20 min to enable adhesion
of wax onto the glass slides.

iii. Immunofluorescence localization of serotonin: Prior to immunolabelling, slides
were dewaxed twice in 100% xylene, for 1 h each, followed by xylene:ethanol (1:1)
and 100% ethanol for 30 min each. Subsequently, sections were rehydrated by passing
through an ethanol gradation series (90%, 85%, 80%, 70%, 50%, 30%, 20% and 10%
ethanol in PBS for 30 min each) and finally PBS (10 min). The deparaffinised
sections were incubated with rabbit polyclonal antibody against serotonin (Abcam,
UK) at a dilution of 1:100. According to the manufacturer’s instruction, primary
antibodies were detected with Histostain-plus kit using LAB-SA detection system
(Invitrogen, USA). The slides were mounted in glycerol and viewed in Axioskop light
microscope.

Estimation of serotonin in basal ends of hypocotyl explants subjected to
pharmacological treatments

Serotonin was analyzed in tissues according to Kang et al. (2007) with minor
modifications. Briefly, 1 g tissue from the basal 6 mm hypocotyl segments (pooled
from several explants subjected to specified treatments till 4 d) was homogenized in
10 mL of methanol (HPLC grade) and centrifuged at 13,500 g for 10 min. The
supernatant was filtered through Millex-LG (Waters, Milford, MA, USA) filter and 2.5 mL (1/4 vol) of water (HPLC grade) was added to the filtered solution. The sample was then passed through a Sep-pak C18 (Waters) cartridge, and the cartridge was washed with 12.5 mL (i.e., same volume as above) of 80% methanol. The pass through and wash solutions were combined and the mixture was evaporated to dryness. The dried extract was then reconstituted in 0.2 mL of 50% methanol. This reconstituted extract was analyzed using reversed- phase HPLC [Waters system (Pump 515, PDA Detector 2998)] using a Waters Symmetry C18 column (length 250 mm and internal diameter 4.6 mm). Isocratic eluent of 5% (v/v) methanol in water containing 0.3% trifluoroacetic acid at a flow rate of 0.8 mL min$^{-1}$ was used and the eluates were detected at 280 nm. Standard curve was generated using serotonin standards (Serotonin hydrochloride, Sigma) at concentrations of 1, 5, 10, 25, and 50 µg mL$^{-1}$.

*All experiments were conducted at least thrice.*