Summary and Conclusions
1. The process of adventitious root (AR) formation consists of three physiologically interdependent phases—induction, initiation, and extension. Induction phase comprises of various molecular and biochemical events but no morphologically visible changes appear during this phase. Formation of multilayered cells and conception of root primordia occurs during the initiation phase. During initiation phase, root primordia exhibit intra-stem growth. Young roots become visible on the stem surface during the extension phase. Using sunflower as a model system, present work has been undertaken to investigate the possible role of NO during IAA-induced adventitious rooting in hypocotyl segments. Since auxin action is principally based on PIN-regulated polar transport of IAA molecules, and PIN proteins are known to exhibit actin-assisted rapid recycling in the target cells, attempts have been made in the present work to find a correlation between auxin transport, actin, and NO, using specific pharmacological agents.

2. AR formation is stimulated as a result of root excision, which possibly reduces the sink size for basipetally translocating substances, leading to their build up in the hypocotyl base. The view gains support from the preliminary experiments, where explants with intact apical meristem exhibited AR formation but those with decapitated apical meristem did not form AR. Root excision could also probably remove the inhibitors of AR being synthesized in roots. Auxin-mediated AR formation in the hypocotyl explants is associated with gradual anatomical changes in the interfascicular parenchyma. During the induction phase, predetermined cells became cytoplasmically dense within 1 d of incubation. Initiation of cell division became evident after 2 d. After 3 d of IAA treatment, tiers of cells became prominent in this region. Few cells of the inner tier exhibited characteristic cell wall thickenings, which proliferated to a greater extent by day 4. These presumably differentiate into vasculature in the developing root primordium. Root primordium, thus initiated after 4 d of IAA treatment, showed a well-developed meristem and procambial tissues.

3. Treatment with IAA (10 µM) elicited two effects on hypocotyl explants in comparison to those subjected to distilled water treatment: 1. Formation of greater number of root initials, 2. Greater extension of the initiated roots. A
response similar to that evoked by IAA is also evident in hypocotyl explants treated with 100 µM of sodium nitroprusside (SNP), which is a donor of nitric oxide. In presence of PTIO (1.5 mM; a specific NO scavenger), complete suppression of AR was evident in sunflower. A combination of PTIO and IAA lead to root initiation only. 1-naphthylphthalamic acid [NPA (10 µM), a blocker of endogenous efflux], blocked AR initiation both by endogenous (distilled water treatment) and exogenous IAA.

4. While no major changes in the polypeptide pattern were evident in the molecular mass above 25 kDa, noteworthy quantitative changes were evident in different treatments in polypeptides with molecular mass between 12-25 kDa. A total of eight such polypeptides have been noted to show quantitative changes. Thus, a polypeptide with estimated kDa of 19.6 shows most prominent abundance in the treatments of NPA, Lat B and CsA inspite of the fact that NPA and latrunculin B (Lat B), an actin depolymerising agent, are inhibitory and Cyclosporin A (CsA, known to inhibit NOS in animal systems) is promotory for AR formation. Thus, the said polypeptide is likely to have a general role in endomembrane recycling/cytoskeleton organization.

5. Coinciding with AR differentiation in sunflower hypocotyl explants, a gradual increase in fluorescence due to endogenously localized nitric oxide (NO) was observed in the interfascicular parenchyma with increasing period of IAA treatment. NO generation in cells exhibiting AR initiation appears to accompany mitotic activity during the initiation phase. Appreciably intense fluorescence due to NO was visualized in the root primordium after 4 d, it being maximum in the apical meristematic cells. This may be attributed to the possible involvement of NO during cell division and differentiation. In the root primordium, NO signal was uniform and symplastic in the epidermal layer of the primordium.

6. Treatment of hypocotyl explants with NPA (10 µM) lead to AR inhibition and complete abolition of NO-associated fluorescence detected by DAF-2DA, indicating that NO acts downstream of auxin during AR formation. In the non-inductive conditions of NPA+IAA, some fluorescence was observed which was quenchable by PTIO. Lat B has been widely used to investigate the function of
actin in gymnosperms, monocotyledonous and dicotyledonous plants. It induces profound changes in the organization of microfilaments while leaving the organization of microtubules unaltered. In the present work, Lat B lead to a negative impact on NO accumulation in the responding cells. Actin depolymerization using Lat B would supposedly lead to substantial inhibition of IAA transport through detection of vesicle movement, which are the carriers of PIN proteins during endomembrane recycling. This results in reduced accumulation of IAA in the basal responding region of the hypocotyl segments. This might explain the observed significant reduction in NO accumulation.

7. Cyclophilins have recently been reported to be a component in auxin regulation of plant growth and development. They interact with the N-terminal region of the GNOM protein, a guanine nucleotide exchange factor (GEF) required for the proper localization of the auxin efflux carrier PIN1. Cyclosporin A (CsA) inhibits NOS activity in animal systems. Compared to the IAA-induced NO fluorescence, intensity of NO fluorescence in root initials of CsA-treated explants was faint. Interestingly, CsA+IAA treatment markedly reduced the intensity of NO-associated symplastic fluorescence in the dividing cells of interfascicular region and the developing root initials. Thus, a potential involvement of cyclophilins in AR formation is apparent from the present work.

8. In order to further investigate the probable involvement of putative NOS in AR formation, tissue homogenates (10,000 g supernatant) from the basal regions of sunflower hypocotyl explants were assayed for total NO content. NO production by putative NOS activity was also estimated. NO content in IAA and distilled water treated explants is more than double than that obtained with treatments inhibitory for AR formation (NPA, Lat B and their combinations with IAA). NO contribution by putative NOS activity also showed a similar pattern. It is, thus, evident that NOS-associated NO production is significantly more in auxin-treated explants. NPA alone, and in conjugation with IAA, lead to a drastic reduction in NO production that appears to be primarily contributed by a decrease in putative NOS activity. Marked reduction in NOS-like activity by NPA and Lat B brings forth the probable involvement of auxin and actin during NO synthesis by
putative NOS activity. NOS is known to be active only when dephosphorylated. The observed inhibition in NO synthesis due to putative NOS activity in CsA-treated explants may be explained as a probable result of CsA-mediated maintenance of phosphorylated, inactive state of putative NOS. A complete inhibition of putative NOS activity in presence of CsA throws light on the presumption about the presence of a NOS-like enzyme. A reduction in NO content and NO due to putative NOS evident in NPA, Lat B and CsA treatments, indicates the involvement of the three components of AR signalling (NO, IAA and actin) and their interaction.

9. The fluorescent probes commonly used for NO detection do not bind directly to NO, but to its other reactive form, like N$_2$O$_3$. DAF does not bind NO directly. It reacts with its oxidized form i.e. N$_2$O$_3$, under oxygenic conditions. Present work reports about a simple, two step synthesis and application of (4-methoxy-2-(1H-napthol[2,3-d]imidazol-2-yl)phenol) [MNIP-Cu] for specific and rapid binding with NO, leading to its detection in plant cells by fluorescence microscopy. MNIP-Cu is non-toxic at the concentrations applicable for its use (10-50 µM), is cell permeable, directly binds NO at its intracellular sites rapidly, and the specificity of fluorescence due to MNIP-NO complex is evident from its quenching by 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO, a well known NO scavenger). In contrast with DAF, which binds with N$_2$O$_3$ (and not NO), MNIP-Cu complexes with NO. Using sunflower (Helianthus annuus L.) hypocotyl segments incubated in adventitious root inducing factor (IAA) and protoplasts isolated from hypocotyl segments subjected to IAA, NPA and SNP treatments, present investigations demonstrate the versatile nature of MNIP-Cu in its applications for NO localization. There is a surge in NO production till the base of the emerging roots. Thus, a differential distribution of NO in the different stages of adventitious rooting is evident. This is the first report on the accumulation of NO in the whole hypocotyl explants subjected IAA treatment for adventitious rooting.

10. Any signal (like IAA) leading to a polarized developmental event, such as AR formation, would very likely affect cytoskeleton organization, in particular actin.
Confocal laser scanning microscopic (CLSM) imaging of fluorescence due to actin upon treatment with Alexa Fluor phalloidin shows contrasting differences between auxin-treated hypocotyl segments and those treated with NPA and Lat B. The basal region of IAA-treated hypocotyl segments responded by exhibiting AR formation, showed enhanced and well distributed actin in the interfascicular cells after 4 days of incubation. Lat B treatment lead to bundling of actin and markedly reduced fluorescence due to actin. NPA leads to inhibition of polar auxin transport, thereby drastically reducing the cellular auxin concentration and consequently leading to observed actin disintegration. PTIO treatment also lead to a drastic actin cytoskeleton breakdown, the fluorescence appearing as a thick fluorescent layer below the cell membrane and bundling of actin, visible as spots of intense fluorescence.

11. Regulation of protein function by NO-mediated post-translational modifications is a recent area of research in plant biology, and is likely to elucidate the mechanism of NO action in regulating many plant processes. Nitration of Y residues to 3-nitrotyrosine has been well characterized in mammals but not much information is available in plants. A new level of regulation of primary metabolism is expected to emerge through post-translational nitration of key enzymes and modification of their catalytic properties. The detection of tyrosine nitrated proteins was examined in sections obtained through the basal regions of hypocotyls subjected to various treatments which were either, inhibitory, partially stimulatory or completely stimulatory for AR formation. From these observations it is evident that 1. Transport of metabolites across the xylem elements coincides with tyrosine nitration of proteins in the concerned cells. 2. The region destined to show root AR initials possesses relatively less tyrosine nitrated protein expression compared to the neighbouring vascular bundles. 3. Root initials preferentially show nuclear localized tyrosine nitrated proteins. 4. The tyrosine nitrated protein expression becomes diffuse and apoplastic during extension phase. Thus, a AR development stage specific distribution and intensification of tyrosine-nitrated proteins is evident in hypocotyl segment exhibiting AR development. A comparison with coomassie stained gels of the separated proteins shows that most abundant anti-nitro tyrosine labelling is evident in proteins
ranging from 25-80 kDa. Noteworthy abundance of tyrosine nitration in treatments triggering adventitious rooting (IAA and SNP) is evident in the protein having a molecular mass close to 25 kDa. Tyrosine nitration is completely absent in presence of NPA (a treatment which suppresses AR formation). Present observations from the in gel labelling experiments are in agreement with the differences observed in spatial localization of tyrosine nitrated proteins observed in the hypocotyl sections. These findings present first report on a possible correlation between AR and tyrosine nitration of proteins.

12. The localized and temporal distribution of reactive oxygen species (ROS) is critical for intercellular and intracellular transduction of ROS signals. ROS-mediated signalling is controlled by a delicate balance between their production and scavenging. The enzymatic defense systems against ROS which lead to neutralizing or scavenging free radicals, include the activity of superoxide dismutase (SOD) and peroxidase (POD). In the recent past, ROS, particularly superoxide and H$_2$O$_2$ have been reported to act as second messengers in various aspects of plant growth and development. Present work reveals elevated activity of SOD in IAA-treated hypocotyl segments. The total soluble protein (10, 000 g supernatant; TSP) shows the expression of two isoforms (no. 2 and 3) in addition to the constitutively present isoforms (1 and 4). Increase in SOD activity in SNP (NO donor) was largely due to enhanced activity of constitutive forms (1 and 4). Thus, IAA and NO seem to promote adventitious root formation by modulating the activities of specific SOD isoforms, indicating the role of H$_2$O$_2$ as a signalling molecule in this morphogenic response. Three POD isoforms were uniformly expressed in two AR promoting treatments (IAA and SNP) and one inhibiting treatment (NPA). Of the two AR promoting treatments, SNP-induced AR response correlates with highest level of total POD activity, followed by IAA. The POD activity is least with NPA treatment. These findings indicate that H$_2$O$_2$ is not only produced in abundance, thereby signalling AR induction as reported in some other plants, it is also scavenged by POD. Thus, a signalling role of H$_2$O$_2$ produced and quickly scavenged is indicated. AR inhibitory treatment (NPA) exhibits least SOD and POD activities, indicating the absence or reduced level of H$_2$O$_2$ as a signalling molecule for AR formation.
13. Architecture of root system is known to be modulated by serotonin concentration. AR inhibiting treatments, such as PTIO (NO scavenger) and SNP+NPA lead to profuse accumulation of serotonin in the cortical cells (brown deposition). The serotonin accumulation was also not evident in the cells of vasculature. NPA (auxin efflux blocker), another treatment blocking AR formation, however, did show serotonin accumulation in some of the cells of vascular tissue. In the three AR promoting treatments (SNP, IAA and PTIO+IAA), profuse serotonin accumulation was evident in the actively dividing cells of root primordium

The quantification data as revealed through HPLC also correlates to the immunohistochemical visualization pattern. The treatment leading to AR initiation (i.e. PTIO+IAA) shows maximum content of serotonin, which shows a sharp decline in treatments leading to AR extension (IAA and SNP), thus showing the possible involvement of serotonin during the early phase of AR formation. Also, the treatments inhibitory for AR formation (PTIO, SNP+NPA, NPA) shows remarkably reduced accumulation on comparison with PTIO+IAA treatment. These observations point out that: 1. Serotonin exhibits differential spatial distribution accompanying AR formation. 2. Its distribution is most abundant in the actively dividing cells of AR primordia. 3. Auxin promotes wider serotonin distribution whereas with auxin efflux blocker (NPA) treatment, serotonin remains confined to vascular bundles, coinciding with AR inhibition.