Introduction
Adventitious roots (AR) are post-embryonic roots known to originate from stem, leaf petiole and non-pericycle tissues of old roots. In young stem, AR commonly arise from the interfascicular parenchyma while in older stem they appear from vascular rays near the cambium. AR formation begins with redifferentiation of predetermined cells which switch from their morphogenetic path to act as mother cells for the initiation of root primordium (Li et al., 2009a). The process of AR formation consists of three physiologically interdependent phases – induction, initiation and extension (Li et al., 2009a). 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. Various environmental and endogenous factors, such as temperature, light, hormones (particularly auxin), sugars and mineral salts, act as cues for promoting redifferentiation of predetermined cells resulting in root induction.

The three phases of AR formation are known to be regulated by alterations in the endogenous level of auxin (Bellamine et al., 1998). A transient increase in auxin concentration has been reported during the induction phase, which is followed by a decrease and again an increase during extension phase (Nag et al., 2001). Auxin transport to and from the responding region is essential for root organogenesis. Acropetal transport of auxin occurs through the vascular cylinder and the basipetal transport takes place through the epidermal and subtending cortical cells (Jones, 1998). Polar transport of auxin from the shoot apical meristem to the rooting region is primarily facilitated by auxin influx (AUX1) and efflux (PIN) transporters. Asymmetric localization of these transporter proteins in the vascular cambium cells is responsible for differential distribution of auxin in a particular zone of cells (Taiz and Zeiger, 2010). Polar auxin transport is known to be inhibited by 1-naphthylphthalamic acid (NPA, a phytotropin). This inhibition is mediated through a binding of NPA molecule to the putative NPA-binding protein (NBP), which is functionally associated with PIN proteins (Morris et al., 2004). Efflux transporters exhibit rapid turnover in the plasma membrane (Morris, 2000). High affinity of NBP for actin filaments
suggests its involvement in the cycling and polar distribution of PIN proteins (Geldner et al., 2001; Munday and Delong, 2001; Munday and Murphy, 2002). Organization of actin filaments is known to be rapidly, reversibly and specifically disrupted by Latrunculin B (Lat B), a macrolide toxin isolated from *Latrunculia magnifica*, a red sea sponge (Spector et al., 1983). Lat B associates with actin monomers in 1:1 ratio, thereby preventing their repolymerization into filaments, resulting in a complete shift from F-actin to G-actin (Walter et al., 2000). Owing to its well understood and simple mode of action and low effective dosage, Lat B has been supplemented with the classic actin-depolymerizing drug cytochalasin D in pharmacological investigations (Gibbon et al., 1999). In the past few years, significant work has been done on NO as a signalling molecule in a variety of plant developmental processes (Moreau et al., 2010). Nitric oxide is known to play a crucial role in root development (Stöhr and Stremlau, 2006).

Although genetic approaches have clarified some discrete aspects of auxin transport and related signal transduction events, not much is known about the molecular mechanisms associated with AR formation. Using various pharmacological agents, a probable signalling cascade (linking NO, cyclic GMP and mitogen-activated protein kinases) for auxin-induced adventitious root formation has been proposed in cucumber seedlings (Pagnussat et al., 2002, 2003, 2004). These observations are, however, devoid of any extensive analysis of the modulation of endogenous NO distribution. NO-mediated regulation of protein function is a recent area of research in plant biology. Very limited number of nitrated proteins with different functions have been identified so far (Aulak et al., 2001; Lozano-Juste et al., 2011). Negi et al. (2010b) isolated a NO-overproducing mutant of tomato in which hyperaccumulation of NO, associated with increase in NOS-like activity, caused diminished growth and delayed flowering. The mode of auxin interaction with NO, resulting in adventitious root formation, still remains unknown.

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. Additionally, effect of Cyclosporin A (CsA), an inhibitor of nitric oxide synthase (NOS) in animal systems (Kleinert et al., 2005), has also been investigated. These specific agents have been used to monitor root initiation at the target sites to decipher a signalling cascade leading to AR formation.

Based on the present understanding of signalling events associated with adventitious rooting, temporal accumulation of endogenous NO in the adventitious root differentiating zone (at various stages of development), has been monitored by fluorescence microscopy, using DAF-2DA for detection of NO, in hypocotyl segments subjected to various pharmacological treatments. These observations highlight the effects of various promoters and inhibitors of AR on the spatial distribution of NO in the responding cells. Estimation of total NO content and NO content due to putative NOS activity has also been undertaken in the hypocotyl segments subjected to various pharmacological treatments known to stimulate or inhibit AR. A comparative confocal microscopic image analysis of the bundling of actin in presence of auxin efflux blocker (NPA) and nitric oxide scavenger [2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO)] has highlighted the common features of their molecular action during AR formation.

Present work also reports about a simple, two step synthesis and application of (4-methoxy-2-(1H-naphtol[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.
Thus, an alternative fluorescent probe for NO detection is proposed for applications in hypocotyls explants and protoplasts for various physiological investigations.

The present work has highlighted auxin-modulated changes in the actin distribution pattern in the interfascicular cells of hypocotyl segments, using a fluorescent probe-Alexa Fluor phalloidin. Recently, Nick et al. (2009) have observed auxin-induced transformation of actin bundles into finer strands and have proposed a self-regulating circuit between polar auxin transport and actin organization. As NO appears downstream of auxin in the signalling pathway (Pagnussat et al., 2003), auxin-mediated actin cytoskeleton maintenance could possibly be modulated by NO. Recently, NO has been reported to modulate actin cytoskeleton in the root apices of maize seedlings (Kasprowicz et al., 2009). From the present investigations, an interaction between IAA, NO and actin reorganization is evident in modulating AR induction, initiation and elongation.

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. Little is known about reactive nitrogen species (RNS) in plant cells as compared to animal systems. One reason could be the difficulty in detecting RNS in biological samples because of their short lifetime as a consequence of their reactivity with other molecules. Chaki et al. (2009) provided first account of the identification of proteins that undergo tyrosine nitration in plants under physiological conditions. They analyzed tyrosine nitration of proteins by LC-MS and also localized nitrotyrosine proteins in sunflower seedling hypocotyls section by CLSM, using antibodies against 3-nitrotyrosine. Hypocotyls derived from sunflower seedling hypocotyls revealed at least six immunoreactive bands in the molecular mass range of 15-68 kDa, which underwent tyrosine nitration (Chaki et al., 2009). In the present work, the detection of tyrosine-nitrated proteins was undertaken 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. On similar lines, detailed analysis of tyrosine nitrated cytosolic proteins from hypocotyl segments subjected to AR promotory (IAA, SNP) and AR inhibiting
(NPA) treatments, has been undertaken by immunolocalization in gel by western blotting. Based on these findings, the significance of protein nitration during AR formation has been highlighted. Lastly, attempts have been made to understand the role of serotonin—a tryptophan derivative, which has been recently found to play a growth regulatory role (Murch et al., 2001). The spatial distribution of serotonin has been monitored by immunohistochemical means to know whether it exhibits any preferential accumulation in response to or corresponding with AR formation. Analysis of serotonin by HPLC has further revealed the quantitative variations in relation with AR formation.