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ORIGINAL ARTICLE

Apoplastic reactive oxygen species mediated escape growth of root during illumination in *Vigna radiata* (L.) Wilczek seedlings

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Abstract

Besides gravity, roots are also guided by light to grow deep into the soil and sensitivity of roots to light is evidently due to presence of photoreceptors like phototropins. Such light-induced root growth (light-escape growth) presumably involves reactive oxygen species (ROS). Present study explores the possibility of ROS action in this event during early seedling growth of *Vigna radiata* based on pharmacological evidences. Germinated (20 h) seeds were incubated in dark or light in presence of general ROS scavenger (propyl gallate), specific scavengers of $O_2^{\cdot-}$ (copper chloride; $CuCl_2$), H_2O_2 [dimethylthiourea (DMTU) and potassium iodide (KI)] and $^{\cdot}OH$ (sodium benzoate) and ROS-producing enzyme inhibitors [zinc chloride ($ZnCl_2$), inhibitor of NADPH oxidase (NOX); diethyldithiocarbamate (DEDTC), inhibitor of superoxide dismutase (SOD) and salicylhydroxamic acid (SHAM), inhibitor of peroxidase]. Light-induced root growth of 3-day seedlings diminished significantly in case of all the treatments suggesting for a positive role of ROS in light-escape growth. This is supported by elevated level of apoplastic ROS in light grown roots as evident from ROS-specific staining [nitroblue tetrazolium chloride (NBT) for $O_2^{\cdot-}$ and 3,3,5,5-tetramethylbenzidine (TMB) for H_2O_2] and spectrophotometric estimation of apoplastic ROS production ($O_2^{\cdot-}$ and H_2O_2). In addition, higher activity of membrane bound NOX (producing $O_2^{\cdot-}$) and apoplastic class III peroxidase (Prx, producing $^{\cdot}OH$) in light grown roots further corroborates the view that apoplastic ROS (initiated with NOX-generated $O_2^{\cdot-}$, which is converted, either spontaneously or by the activity of SOD, to H_2O_2 and further metabolized by Prx to $^{\cdot}OH$ that participates in cell wall relaxation required for growth) is instrumental in light-escape growth of roots.

Keywords Light-escape root growth · NADPH oxidase · Peroxidase · Reactive oxygen species · Superoxide dismutase

Introduction

Light, being one of the key environmental resources essential for plant growth and development, regulates a wide array of plant processes ranging from momentary responses like chloroplast movements to permanent developmental alterations (Yokawa et al. 2011; Paddock et al. 2012; Majumdar and Kar 2016; Wada and Kong 2018). Interestingly, plant organs demonstrating light-dependent developmental changes (photomorphogenesis) include even roots, which are naturally programmed to grow into the depth of the soil i.e., away from light (Gelderen et al. 2018). Further, presence of diverse sensory photoreceptors and their significant expression in root indicates that photon absorption is a frequent event in these tissues (Galen et al. 2007a). Perception of incident light and subsequent signaling helps in achieving the prominent directionality in root growth pattern (negative phototropism) as indicated by penetration of root system into the deeper soil layers away from the light source (Silva-Navas et al. 2016). In accordance with their natural photophobic character, roots show typically accelerated growth under illuminated condition (Yokawa and Baluska 2016). Although differing in signaling, modes and magnitude, so called "light-escape growth" of root may be considered analogous to the shade avoidance system operating at the aboveground portion of plants. Therefore, additional protection

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from light (beyond photo-negative vector) is obtained from this mechanism that facilitates augmentation of directional root growth (Burbach et al. 2012) thereby acting as a guidance mechanism for roots in addition to gravitropism. However, availability of light below the soil surface is a matter of curiosity and is being studied for long (Neel 1948; Perkins 1963; Woolley and Stoller 1978).Reports published over the decades have shown that light can variably penetrate the soil surface depending on the soil texture as well quality and fluence of light with red/far red light reaching deeper than blue light (Mo et al. 2015). Apart from direct infiltration through soil, light can also be transmitted (or piped) to the photoreceptors present in the root by xylem elements utilizing the property of internal reflectance rendering these tissues analogous to the optical fiber cables (Mandoli and Briggs 1982; Galen et al. 2007a).

Similar augmentation of root growth has also been noted in response to drought. Mild water stress is reported to promote root growth (as an adaptive mechanism) enabling them to explore new depths of soil in search of water (Rodrigues et al. 1995; Sharp et al. 2004; Das and Kar 2017). Interestingly, Galen et al. (2007b) have noted that PHOT1 (phototropin 1; specific blue light photoreceptor) is indispensable for root growth under drought stress as *phot1* mutants were growing at half the rate that of control plants. They hypothesized that PHOT1 and other plant photoreceptors are pleiotropic in nature and control plant processes across organs simultaneously e.g., *phot1* mutants showed 35% reduction in transpiration rate along with stunted root growth. Moreover, such drought stress induced root growth promotion was found to be regulated by ROS (Das and Kar 2017) as was also reported in the case of light-escape growth mediation (Yokawa and Baluska 2016). Thus, it appears that both light-escape and drought-induced root growth have common pathway involving phototropins and ROS.

Contrary to their distinct deleterious roles, beneficial involvements of ROS in most of the plant growth and developmental processes are being steadily identified over time (Garg and Manchanda 2009; Mittler 2017). Regulation of root growth and development is a well reported exemplar of ROS-signaling in plants (Liszkay et al. 2004; Majumdar and Kar 2018, 2019). ROS-mediated cellular growth has been envisaged to occur through a pathway where NOX-derived apoplastic superoxide ($O_2^{\cdot -}$) is first converted to hydrogen peroxide (H_2O_2) by spontaneous reactions or through SOD, which is then acted upon by class III peroxidase (Prx) producing $^{\cdot}OH$ radical effecting cell wall relaxation through $^{\cdot}OH$-mediated cleavage of cell wall polysaccharides. This pathway has not only been reported to regulate root growth, but also found to be operative in case of positive geotropism or gravitropic bending too (Singh et al. 2017). Apparently, other environmental cue-dependent growth mechanics prevailing in roots are also likely to be under ROS regulation. In line with the idea, it is already reported that intracellular ROS accumulation occurring as a result of illumination of root influences light-escape growth of root in *Arabidopsis* (Yokawa et al. 2011; Yokawa and Baluska 2016). However, exact roles of ROS and the specific ROS-generating enzymes, like NOX and Prx in light-escape root growth have not been explored. In the present investigation attempts have been made to explore the possible involvement of NOX and Prx activities and resultant accumulation of ROS in apoplastic space in light-escape growth of root.

Materials and methods

Plant material, incubation and growth measurement

Mung bean [*Vigna radiata* (L.) Wilczek var. B1] seeds were collected from Pulses Research Center, Baharampur, West Bengal and used as experimental material. At first seeds were surface sterilized by 1% sodium hypochlorite solution, followed by repeated rinsing in distilled water and finally incubated on Whatman no. 1 filter paper in the presence of adequate distilled water placed in a 9 cm diameter Petri dish and kept in a seed germinator at 30 °C (± 2 °C). After 20 h, germinated seeds were transferred to Whatman no. 1 filter papers soaked with distilled water (for control) or test solutions (for treatment set) kept in transparent (for light set) and black coated (for dark set) plastic boxes and incubated in a Plant Growth Chamber (Fitotron, Weisstechnik, Germany). Light sets were maintained in a 16 h light (photon flux density of 150 µmol/m^2/s) and 8 h dark cycle whereas the dark sets kept in continuous dark condition at 70% relative humidity and a temperature cycle of 35 °C(day) and 30 °C (night). After 72 h of incubation, lengths of 10 roots were taken each from control (dH_2O) as well as from different treatment sets.

Pharmacological treatments

To assess the roles of ROS in light escape growth of root, germinated seeds were incubated in the following test solutions for pharmacological studies: propyl gallate [PG, general ROS scavenger (Zhang and Kirkham 1996); 1 mM], copper chloride [$CuCl_2$, $O_2^{\cdot -}$ scavenger (Liszkay et al. 2004); 0.5 mM], zinc chloride [$ZnCl_2$, inhibitor of NOX (Liszkay et al. 2004); 1 mM], dimethylthiourea [DMTU, H_2O_2 scavenger (Causin et al. 2012); 5 mM], potassium iodide [KI, H_2O_2 scavenger (Causin et al. 2012); 5 mM], diethyldithiocarbamate [DEDTC, inhibitor of SOD (Lushchak et al. 2006); 5 mM], sodium benzoate [NaBz, hydroxyl radical scavenger (Georgiou et al. 2000); 1 mM] and salicylhydroxamic acid [SHAM, inhibitor of Prx (Causin et al. 2012); 1 mM]. In our

earlier studies on seed germination, post-germination axis and root growth of *Vigna radiata* seedlings these chemicals have been used as pharmacological agents for their specific scavenging and inhibitory properties (Singh et al. 2014, 2015; Majumdar and Kar 2018, 2019).

In vivo accumulation of $O_2^{\cdot -}$

$O_2^{\cdot -}$ accumulation was studied by following the method of Liszkay et al. (2004) with few modifications. Both light and dark grown roots were stained by $O_2^{\cdot -}$ specific stain, NBT (0.5 mM) in sodium phosphate buffer (50 mM, pH 6.8). After 30 min of incubation, roots were washed with distilled water and photographs were taken.

In vivo accumulation of H_2O_2

H_2O_2 accumulation was monitored by following method of Das and Kar (2017). Light and dark grown roots were stained by incubating with H_2O_2 specific stain, TMB (1 mM) for 30 min. After incubation roots were washed with distilled water and photographs were taken.

Spectrophotometric estimation of apoplastic $O_2^{\cdot -}$ production

Extracellular $O_2^{\cdot -}$ production in roots was measured spectrophotometrically following the method of Misra and Fridovich (1972) and Liszkay et al. (2004) with few modifications. Excised roots (light and dark grown) were kept in distilled water for 10 min for release of wound-induced $O_2^{\cdot -}$ and then transferred to a bathing medium comprising of 500 µL sodium phosphate buffer (50 mM, pH 6.8), 250 µL distilled water and 250 µL epinephrine (2 mM) and incubated in dark at 37 °C in a shaker (REMI CM-101 PLUS). After 45 min of incubation, absorbance of bathing medium was measured by UV–Vis Spectrophotometer (Systronics, India) at 480 nm. $O_2^{\cdot -}$ production was calculated using an extinction coefficient of 4020 M^{-1} cm^{-1} and expressed as nM g^{-1} tissue.

Spectrophotometric estimation of apoplastic H_2O_2 production

Extracellular H_2O_2 production in roots was measured spectrophotometrically following the method of Gay and Gebicki (2000), Minibayeva et al. (2009) and Moothoo-Padayachie et al. (2016) with few modifications. As mentioned above, excised light and dark grown roots were kept in distilled water for 10 min before incubating in bathing medium. Bathing medium was comprised of one part of Reagent A (25 mM $FeSO_4$, 25 mM $(NH_4)_2SO_4$, 2.5 M H_2SO_4) and 100 parts of Reagent B (125 µM xylenol orange, 100 mM sorbitol). After 2 h of incubation in dark at 25 °C under shaking, absorbance of the bathing medium was measured spectrophotometrically at 560 nm. H_2O_2 production was calculated using a standard curve with known concentrations of H_2O_2 and expressed as ng g^{-1} tissue min^{-1}.

In-gel assay of NADPH oxidase (NOX)

Isolation of membrane fraction

The membrane fraction from root tissue was isolated following the method of Hejl and Koster (2004) with some modifications. Tissues from both light and dark grown roots were homogenized separately in 1 mL extraction buffer containing sodium phosphate buffer (50 mM, pH 6.8), sucrose (250 mM), EDTA (ethylene diamine tetraacetic acid; 3 mM), DTT (dithiothreitol; 1.25 mM), PMSF (phenyl methyl sulphonyl fluoride; 1 mM) in cold condition and the homogenates were centrifuged at 10,000 rpm for 10 min at 4 °C (REMI C-24 PLUS). Supernatants were collected and again centrifuged at 16,000 rpm for 45 min at 4 °C. Then pellets were resuspended in 50 µL resuspension buffer containing sodium phosphate buffer (50 mM, pH 6.8), sucrose (250 mM), triton X-100 (0.5%), DTT (1 mM) and PMSF (1 mM) and kept in ice for 1 h. After that, supernatants were again centrifuged at 16,000 rpm for 45 min. Final supernatants were collected and used as enzyme sources for NOX assay.

In-gel native PAGE assay of NOX

From the above collected supernatants, NOX activity was determined in 7.5% vertical native PAGE at 4 °C. After determining the protein content by Bradford reagent (Bradford 1976) 15 µg protein each from both light and dark grown root samples was loaded in separate lane with loading buffer in a 5:1 ratio and run. NOX activity was detected in gel slab using NBT stain (Sagi and Fluhr 2001; Frahry and Schopfer 2001 with modifications). Staining was done by incubating the gel slab in 30 mL Tris HCL buffer (10 mM, pH 7.4) containing NBT (0.5 mM), NADPH (0.2 mM), $MgCl_2$ (0.1 mM), $CaCl_2$ (1 mM) and sodium azide (NaN_3, 1 mM) in a gel rocker for 30 min. Violet formazan bands appeared due to reduction of NBT by NOX-generated $O_2^{\cdot -}$. Presence of sodium azide (NaN_3) eliminated any possible activity of Prx (Carter et al. 2007). In a separate experimental set, diphenylene iodonium (DPI, 0.2 mM) was added in the assay medium for confirmation of NOX enzyme activity in the gels (Supplementary Figure S2).

Spectrophotometric assay of apoplastic Prx activity

Apoplastic Prx activity was determined following the method of Singh et al. (2015). Light and dark grown roots

were incubated in bathing medium containing of 250 µL phosphate buffer (50 mM, pH 6.8), 250 µL H_2O_2 (10 mM) and 250 µL pyrogallol (10 mM) for 2 min at 25 °C in darkness. The reaction was terminated by removing roots from bathing medium immediately followed by addition of 250 µL H_2SO_4 (5%). The zero time control set contained 5% H_2SO_4 from the beginning. The quantity of oxidized pyrogallol (purpurogallin) was determined by measuring absorbance at 430 nm in a UV–VIS spectrophotometer. The activity of enzyme was calculated using the formula of Fick and Qualset (1975).

Statistical analysis

All the experiments were repeated at least three times and in each case three replicates were used except for root growth experiments where 10 seedlings were used for length measurements. The accumulated data were analyzed using Microsoft Excel 2007 software. Definite software-specific formulae were used to calculate mean, standard error (SE) and standard deviation (SD) values. With the help of in-built chart tool, data were plotted as vertical bar graphs accompanied by standard error (SE) of the mean. Data were further analyzed by appropriate single factor ANOVA and post hoc comparisons were done with Tukey's HSD to determine statistically significant differences among individual treatments at $P < 0.05$ level following Singh et al. (2015). Asterisk indicates significant difference among means at $P < 0.05$ level between control and treatment sets.

Results

In the present study, roles of ROS with new insight into apoplastic ROS metabolism in light escape growth of root have been examined on the basis of physiological and biochemical parameters. Significant enhancement of root length was observed in light grown root over the dark control as presented in the photographs of seedlings (Fig. 1a, e) as well as in the bar graph (Fig. 1f). Role of ROS in such light-induced root growth was determined by observing the effects of standardized concentrations of different pharmacological treatments (ROS scavengers and ROS producing enzyme inhibitors). Although, as a general ROS scavenger, PG did not inhibit significantly light escape root growth, application of $CuCl_2$ ($O_2^{\cdot -}$ scavenger) and $ZnCl_2$ (inhibitor of $O_2^{\cdot -}$ producing enzyme, NOX) resulted in remarkable inhibition of root growth to a minimum in light (Fig. 1b, c, d, f). Similarly, effect of DMTU and KI (H_2O_2 scavenger) and DEDTC (inhibitor of H_2O_2 producing enzyme, SOD) on light escape growth of roots has been displayed along with light and dark controls in the photographs (Fig. 2a–e) and in bar graphs (Fig. 2f). Clearly, treatment with DMTU and KI retarded root growth differentially (DMTU being

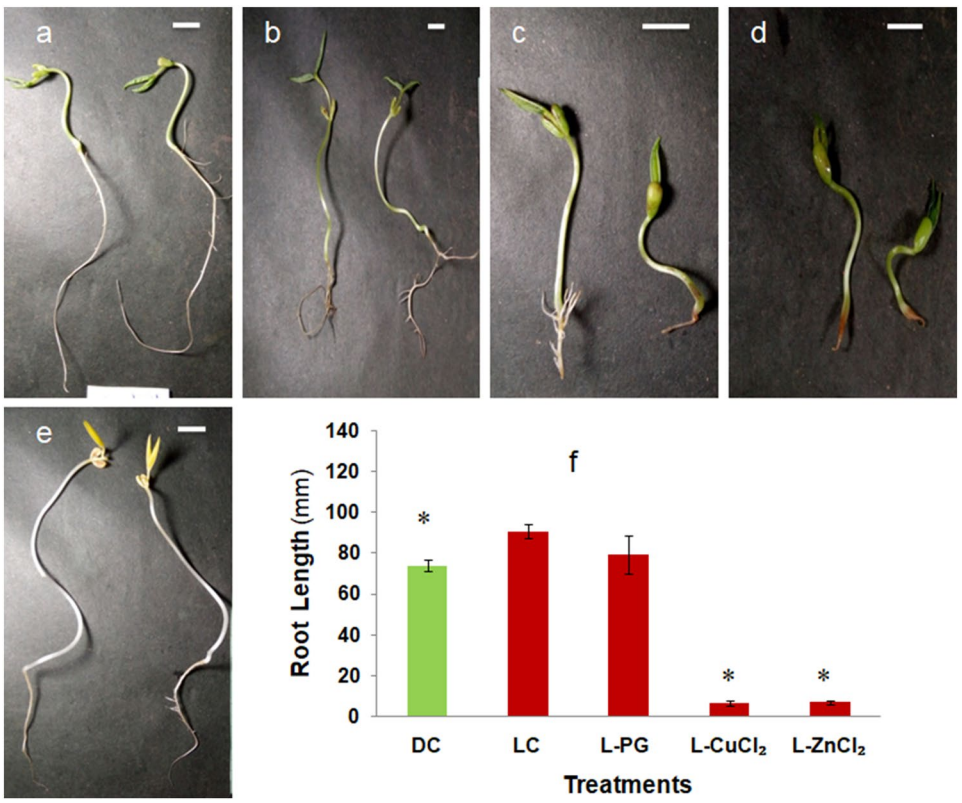

Fig. 1 Role of ROS (in general) and $O_2^{\cdot -}$ in light-escape growth of root: photographs are representatives of 10 replicates of 72 h old seedlings of *Vigna radiata* grown in plant growth chamber in presence of light (**a**–**d**) on filter paper moistened with distilled water (**a**), 1 mM propyl gallate (**b**), 0.5 mM copper chloride (**c**), 1 mM zinc chloride (**d**) along with a dark control (on filter paper moistened with distilled water) (**e**). **f** Root length of the seedlings (average of 10 replicates) grown in distilled water (DC: dark control, LC: light control) and in presence of 1 mM propyl gallate (L-PG), 0.5 mM copper chloride (L-$CuCl_2$), 1 mM zinc chloride (L-$ZnCl_2$). Standard error is presented as vertical bars. Data were subjected to one way ANOVA. Asterisk indicates significant difference among means at the $P < 0.05$ level between control and treatment sets. Bar in case of all photographs is 1 cm

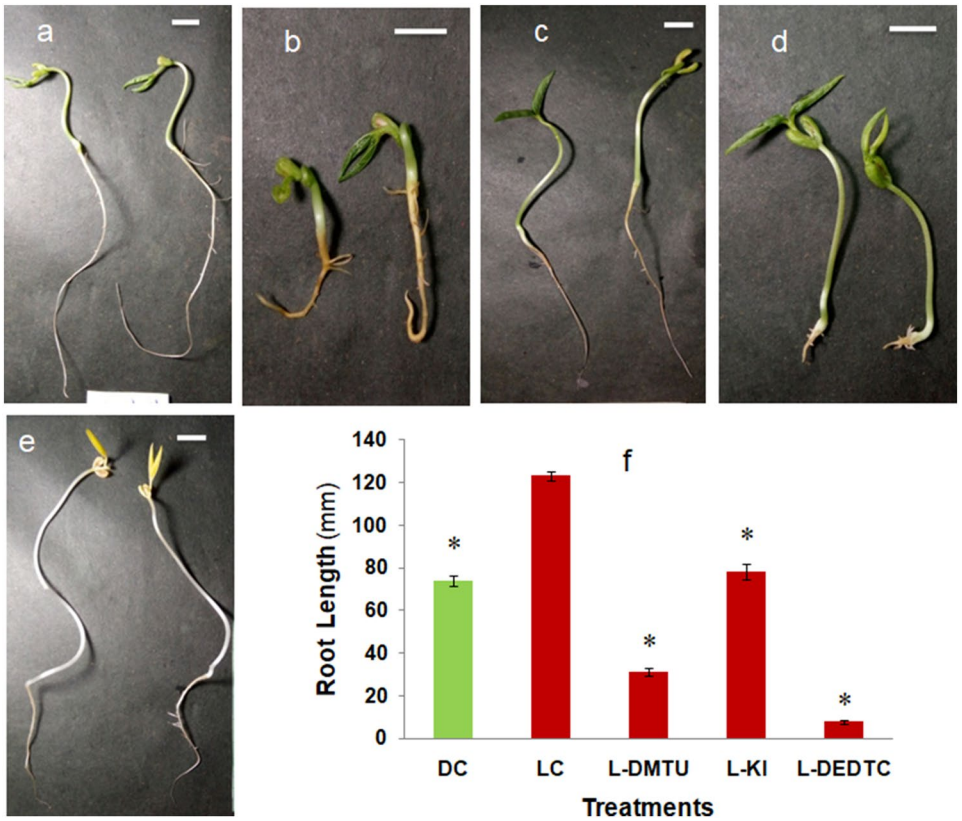

Fig. 2 Role of H_2O_2 in light-escape growth of root: photographs are representatives of 10 replicates of 72 h old seedlings of *Vigna radiata* grown in Plant Growth Chamber in presence of light (**a**–**d**) on filter paper moistened with distilled water (**a**), 5 mM dimethylthiourea (**b**), 5 mM potassium iodide (**c**), 5 mM diethyldithiocarbamate (**d**) along with a dark control (on filter paper moistened with distilled water) (**e**). **f** Root length of the seedlings (average of 10 replicates) grown in distilled water (DC: dark control, LC: light control) and in presence of 5 mM dimethylthiourea (L-DMTU), 5 mM potassium iodide (L-KI), 5 mM diethyldithiocarbamate (L-DEDTC). Standard error is presented as vertical bars. Data were subjected to one way ANOVA. Asterisk indicates significant difference among means at the $P < 0.05$ level between control and treatment sets. Bar in case of all photographs is 1 cm

more effective than KI), while that with DEDTC inhibited growth to a great extent. Next, seedlings were treated with Na-Benzoate (OH·scavenger) and SHAM (inhibitor of Prx, an enzyme producing OH·), effect of which on root growth in light has been presented in the photographs (Fig. 3a–d) as well as in bar graph (Fig. 3e). Photograph clearly shows that root length was more in light grown seedling (Fig. 3a) than in dark grown control (Fig. 3d) and treatments with both Na-Benzoate and SHAM strongly retarded root growth (Fig. 3b, c, respectively). Bar graph (Fig. 3e) also reflects the same trend where root length in treated seedlings was almost one-third of that of the light control.

Apoplastic accumulation of $O_2^{\cdot-}$ in roots of seedlings grown in light and darkness (control) was assessed by staining with NBT (specific for $O_2^{\cdot-}$) for visual effect and by estimating $O_2^{\cdot-}$ level using epinephrine in the bathing medium. It was found that roots grown in light took more intense violet color (Fig. 4a) following NBT staining than the dark grown roots (Fig. 4b). This is also true for data on $O_2^{\cdot-}$ level in the bathing medium where the level was significantly higher in light grown roots relative to dark grown samples (Fig. 4c). Apoplastic localization of $O_2^{\cdot-}$ was further evident from transverse section (TS) of roots as cell walls took the distinct dark blue/violet colour of NBT and cytosol remained colorless (Supplementary Figure S1a, b). In case of assessment of H_2O_2 accumulation in the apoplast, there was no drastic difference in blue coloration of root due to staining with TMB (specific for H_2O_2) between light (Fig. 5a) and dark grown roots (Fig. 5b). However, spectrophotometric estimation of apoplastic H_2O_2 production using xylenol orange in the bathing medium showed significantly enhanced production of H_2O_2 (more than double) in light grown roots than in dark grown roots (Fig. 5c). It was observed in TS of roots that cell walls (not cytosol) were again distinctly stained with TMB supporting apoplastic origin of H_2O_2 (Supplementary Figure S1c, d).

Activity of membrane bound NOX, one of the primary apoplastic $O_2^{\cdot-}$ producing enzymes, was assessed by in-gel native PAGE assay, which showed distinctly higher activity in light grown roots than in dark grown roots as reflected by intense uppermost band (Fig. 6a). Inclusion of NaN_3 in the assay medium eliminated any possible Prx activity. Complete absence of bands in gels, which were incubated in assay medium supplemented with DPI, confirmed that the violet formazan bands appeared as a result of NOX activity only (Supplementary Figure S2). On the other hand, activity of apoplastic Prx, assayed spectrophotometrically (Fig. 6b), was found to be higher in light grown roots than in dark grown roots (Fig. 6b).

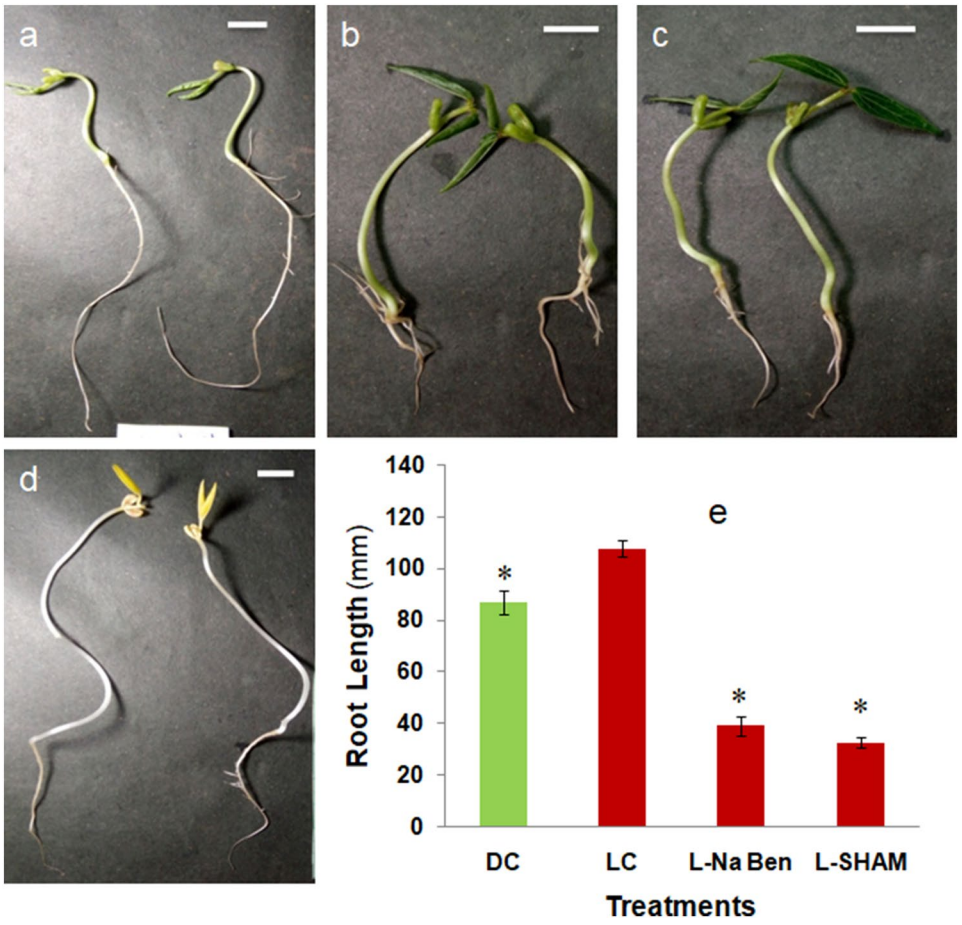

Fig. 3 Role of hydroxyl radical in light-escape growth of root: Photographs are representatives of 10 replicates of 72 h old seedlings of *Vigna radiata* grown in Plant Growth Chamber in presence of light (**a**–**c**) on filter paper moistened with distilled water (**a**), 1 mM sodium benzoate (**b**), 1 mM salicylhydroxamic acid (**c**) along with a dark control (on filter paper moistened with distilled water) (**d**). **e** Root length of the seedlings (average of 10 replicates) grown in distilled water (DC: dark control, LC: light control) and in presence of 1 mM sodium benzoate (L-Na Ben), 1 mM salicylhydroxamic acid (L-SHAM). Standard error is presented as vertical bars. Data were subjected to one way ANOVA. Asterisk indicates significant difference among means at the $P < 0.05$ level between control and treatment sets. Bar in case of all photographs is 1 cm

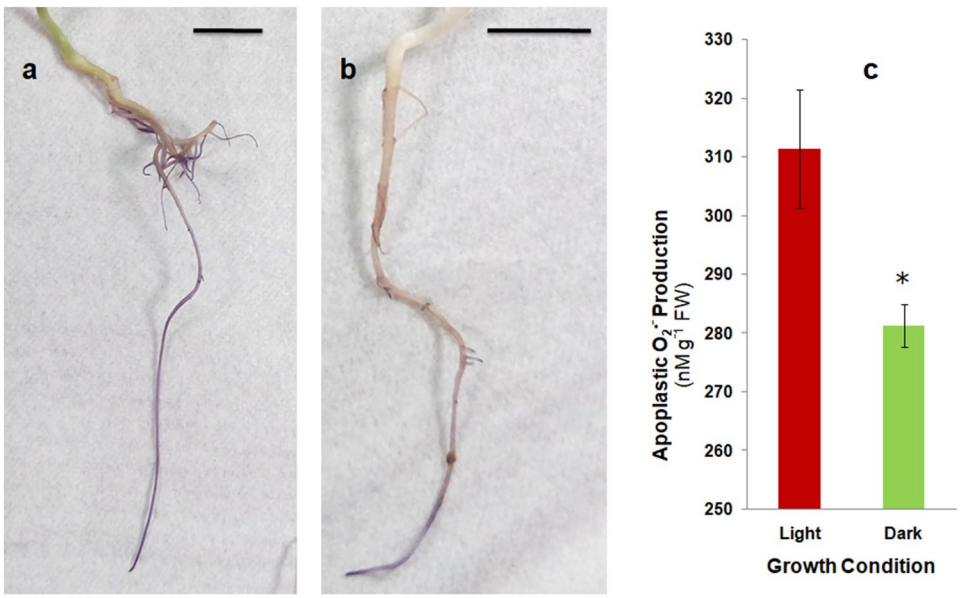

Fig. 4 Staining and estimation for apoplastic $O_2^{\cdot-}$ production during light escape growth of root: roots were stained with Nitroblue tetrazolium chloride (**a**, **b**) for in vivo accumulation of apoplastic $O_2^{\cdot-}$. Production of apoplastic $O_2^{\cdot-}$ (**c**) was estimated spectrophotometrically in both light and dark grown roots. Photographs are representatives of 10 replicates of 72 h old seedlings of *Vigna radiata* grown in plant growth chamber in light (**a**) and dark (**b**) condition. Data were subjected to one way ANOVA. Asterisk indicates significant difference among means at the $P < 0.05$ level between light and dark grown sets. Bar in case of all photographs is 1 cm

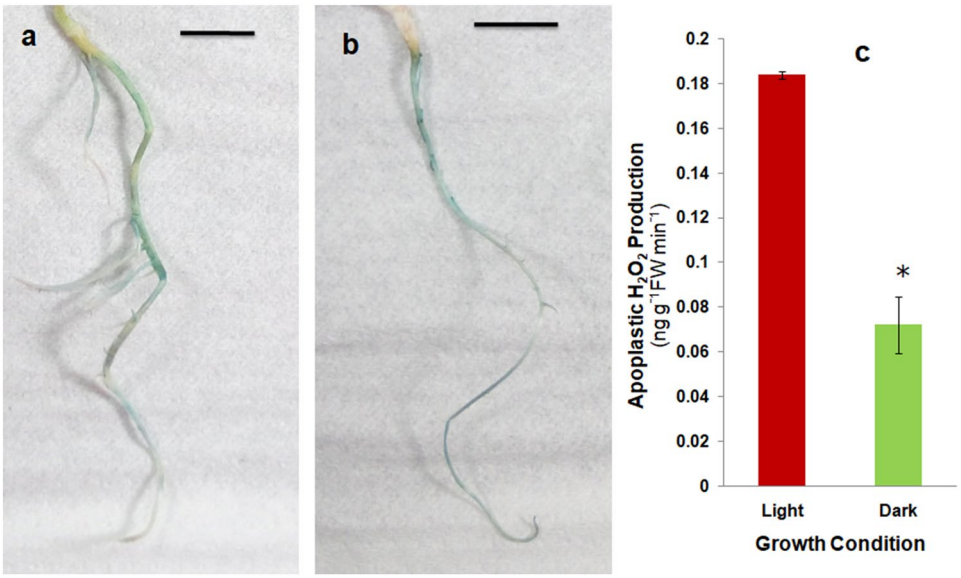

Fig. 5 Staining and estimation for apoplastic H_2O_2 production during light escape growth of root: roots were stained with 3,3′,5,5′-Tetramethylbenzidine (**a**, **b**) for in vivo accumulation of apoplastic H_2O_2. Production of H_2O_2 (**c**) was estimated spectrophotometrically in both light and dark grown roots. Photographs are representatives of 10 replicates of 72 h old seedlings of *Vigna radiata* grown in Plant Growth Chamber in light (**a**) and dark (**b**) condition. Data were subjected to one way ANOVA. Asterisk indicates significant difference among means at the $P<0.05$ level between light and dark grown sets. Bar in case of all photographs is 1 cm

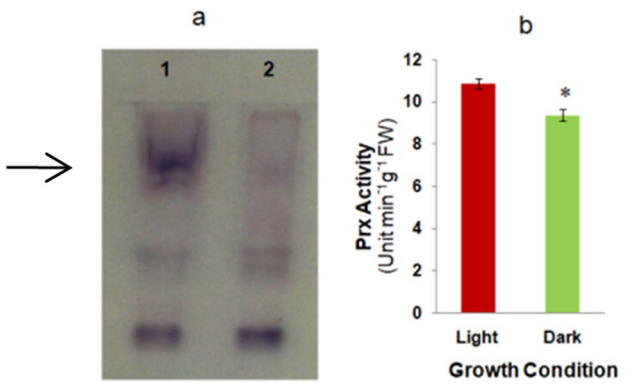

Fig. 6 Involvement of membrane bound NOX and apoplastic Prx in light-escape root growth: native PAGE assay of membrane bound NOX of both light grown (lane 1) and dark grown (lane 2) roots of 72 h old seedlings of *Vigna radiata* (**a**). Activity of apoplastic Prx in both light and dark grown roots of 72 h old seedlings of *Vigna radiata* (**b**). Data were subjected to one way ANOVA. Asterisk indicates significant difference among means at the $P<0.05$ level between light and dark grown sets

Discussion

Plants show flexible patterns of growth for adjustment to the local environment that ultimately help to survive despite adverse situation. While shoot growth is retarded in most of the cases, interestingly root growth is augmented under stresses, like mild drought condition (Das and Kar 2017), light exposure (Yokawa et al. 2011) and nitrogen-deficient condition of soil (Qin et al. 2019). This is in complete agreement with roots' indeterminate growth pattern which enables them to continuously explore new areas of soil for water and nutrients. Having the transition zone (that acts as a hub of signalling responses), roots are able to accelerate or deter growth rate with regard to environmental cues. On the other hand, recent studies have revealed that ROS are involved in, apart from deleterious effects during stress, (redox) signal integration and decision making under both physiological as well as stressed conditions (Causin et al. 2012; Černý et al. 2018; Foyer 2018; Majumdar and Kar 2020). Besides, involvement of ROS in cell expansion (through cleavage of wall polysaccharides) depicts their pivotal role directly in cellular growth (Schopfer 2001; Airianah et al. 2016). Therefore, it may be implicated that root growth induced under environmental cues is mediated by ROS, as was evident in case of enhanced root length under water stress associated with apoplastic accumulation of ROS (Das and Kar 2017). Similarly, perception of incident light and subsequent signaling enables the achievement of prominent directionality in root growth pattern (negative phototropism) which necessarily promotes root to dive into deeper soil to grow away from the light source (Silva-Navas et al. 2016). It has been reported that intracellular ROS accumulation influences escape growth of illuminated root in *Arabidopsis* (Yokawa et al. 2011). Present study with 72 h old seedlings of *Vigna radiata* also demonstrates light-induced root

growth (light escape growth) and a definite role of ROS accumulation, particularly in the apoplastic space, in such light escape growth of root. In skotomorphogenic organs like root, where chloroplasts are not available, mitochondria, peroxisome and apoplast serve as the primary sources of ROS. The apoplastic ROS cascade or network (being constituted by NOX, Cu/Zn SOD, and Prx) has extensively been reported to be involved in cellular signal perception and transduction mechanisms. Interestingly, it has been hypothesized that, as an example of retrograde signalling, apoplastic ROS triggers the production of ROS by other cellular organelles leading to regulation of nuclear gene expression (Padmanabhan and Dinesh-Kumar 2010; Shapiguzov et al. 2012). Moreover, apoplastic ROS, a minor fraction of total cellular ROS, accumulates because of low redox buffer state of the cell wall that creates a condition suitable for signaling (Podgorska et al. 2017).

Thus, treatments with specific $O_2^{\cdot -}$ scavenger ($CuCl_2$)and NOX inhibitor ($ZnCl_2$) (Schopfer et al. 2002; Liszkay et al. 2004; Singh et al. 2014; Majumdar and Kar 2016) severely inhibited light induced root length promotion (Fig. 1) indicating the involvement of $O_2^{\cdot -}$ in light escape growth of illuminated roots. On the other hand, involvement of H_2O_2 was also established as H_2O_2 scavengers (DMTU and KI) and inhibitor of H_2O_2 producing enzyme, SOD (DEDTC) inhibited significantly light-induced root length promotion (Fig. 2). Roles of apoplastic $O_2^{\cdot -}$ and H_2O_2 were further corroborated by data on staining with NBT (specific for $O_2^{\cdot -}$) and TMB (specific for H_2O_2) as well as spectrophotometric estimation of apoplastic $O_2^{\cdot -}$ and H_2O_2 production (Figs. 4, 5, S1).

Plasma membrane located NOX is an important ROS producing enzyme, which produces $O_2^{\cdot -}$ and initiates the ROS cascade in apoplastic space (Kar 2011; Singh et al. 2015; Das and Kar 2017; Podgorska et al. 2017). That NOX is involved in light escape growth of roots of *V. radiata* is evident by higher activity in light grown roots as revealed by in-gel native PAGE assay of membrane bound NOX (Fig. 6a). It may be speculated that in apoplast, PM-located NOX mediated production of $O_2^{\cdot -}$ subsequently converted into H_2O_2 by SOD (Singh et al. 2015), although pH-dependent Prx, germin-like oxalate oxidases and amine oxidase are also different possible sources of H_2O_2 in apoplast (Kar 2011; Podgorska et al. 2017).

Another important enzyme in apoplastic ROS metabolism is class III Prx, which is also a source of $O_2^{\cdot -}$ or H_2O_2 via oxidative cycle or produces $OH^{\cdot}$ from H_2O_2 and $O_2^{\cdot -}$ in hydroxylic cycle in apoplast (Podgorska et al. 2017). Interestingly, activity of apoplastic Prx was also higher in light grown roots than in dark grown roots (Fig. 6b). A definite role of $^{\cdot}OH$ in cell wall relaxation required for cell extension growth through cleavage of wall polysaccharides has been established in case of growth including seed germination and seedling root growth (Singh et al. 2015; Tsukagoshi 2016; Airianah et al. 2016; Majumdar and Kar 2019). Thus it seems quite likely that Prx is also playing a similar role in case of light escape growth of *Vigna radiata* roots. This is supported by the observation that treatments with $^{\cdot}OH$ scavenger (sodium benzoate) and Prx inhibitor (SHAM) severely inhibited light induced root length promotion (Fig. 3).

Conclusion

It appears from the study that apoplastically generated ROS and its metabolism have been playing an important role in the process of escape growth of roots exposed to light. Plasma membrane located NOX, activity of which increased in light-grown roots, seemingly plays a pivotal role by producing $O_2^{\cdot -}$, which is converted to H_2O_2 in the cell wall (apoplast) initiating the ROS cascade. Accumulation of these ROS in the cell wall is evident by specific staining and quantitation of $O_2^{\cdot -}$ and H_2O_2in the apoplast. H_2O_2 may further be converted to $^{\cdot}OH$ by the apoplastic Prx leading to extension growth through cell wall relaxation. Disruption of such apoplastic ROS cascade by using different ROS scavengers and ROS-producing enzyme inhibitors resulted in retarded light-escape root growth corroborating the involvement of ROS cascade in the process. In spite of being a key phenomenon for plants' survival, light-escape growth of roots has received little attention so far and requires further in-depth studies to explain light sensitivity of roots in detail.

Author contribution statement RKK envisaged the study. TD, SD, AM and RKK designed the work. TD and SD performed the experiments. AM performed the statistical analyses. TD, SD, AM and RKK wrote the article.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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