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ORIGINAL PAPER

Accumulation and scavenging of reactive oxygen species and nitric oxide correlate with stigma maturation and pollen–stigma interaction in sunflower

Basudha Sharma · S. C. Bhatla

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Abstract During the course of stigma development in sunflower (bud, staminate and pistillate stages), correlation is evident among the accumulation of reactive oxygen species (ROS), nitric oxide (NO), and the activities of ROS scavenging enzymes [superoxide dismutase (SOD) and peroxidase (POD)]. Confocal image analysis shows a gradual increase in ROS and NO accumulation in the stigmatic papillae, which may provide immunity to the developing stigma and function as signalling molecules. A novel, NO-specific probe (MNIP-Cu) has been employed for the detection and quantification of intracellular NO. Mn-SOD (mitochondrial) and Cu/Zn-SOD (cytoplasmic) exhibit differential expression during the staminate stage of stigma development. An increase in total SOD activity at the staminate stage is followed by a peak of POD activity during pistillate stage, thereby indicating the sequential action of the two enzymes in scavenging ROS. An increase in the number of POD isoforms is observed with the passage of stigma development (from three at bud stage to seven at pistillate stage), and two POD isoforms are unique to pistillate stage, thereby highlighting their role in ROS scavenging mechanism. ROS and NO accumulation exhibit reverse trends during pollen–stigma interaction.

Keywords Helianthus annuus · Nitric oxide · Peroxidase · Pollen · Pollen-stigma interaction · Superoxide dismutase · Stigma development

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B. Sharma · S. C. Bhatla (✉)
Laboratory of Plant Physiology and Biochemistry, Department of Botany, University of Delhi, North Campus, Delhi 110007, India
e-mail: bhatlasc@gmail.com

Introduction

Stigma receptivity refers to its phase development coinciding with the ability to support pollen adhesion on the stigma surface, followed by pollen hydration and subsequent formation of pollen tube (Sanchez et al. 2004). A number of biomolecules are required to enable pollen adhesion, hydration and germination on the stigma surface. Recently, it has been reported that reactive oxygen species (ROS) and nitric oxide (NO) are involved in the initial signalling events between stigma and pollen (Allen et al. 2011; McInnis et al. 2006a; Zafra et al. 2010; Serrano et al. 2012b). Superoxide is the first reduction product of the ground state oxygen (O_2). Due to their negative charge, superoxides are unstable and are not able to diffuse through the cell membrane, thereby acting as poor signalling molecules (Mittler et al. 2011). Hydrogen peroxide (another ROS form) is a stable molecule, selective in activity and is diffusible through cell membranes across aquaporins, thus facilitating its role as a signalling molecule. H_2O_2 mediates responses against biotic and abiotic stresses, such as pathogen attack (McInnis et al. 2005), wounding (Orozco-Cárdenas et al. 2001), water deficit (Jubany-Marí et al. 2010), salinity (Miller et al. 2010), alterations in phytohormone levels (such as ABA) by systemic acquired resistance (SAR) (Zhang et al. 2001), hypersensitive resistance (HR) and programmed cell death (PCD) (Gadjev et al. 2008). It can interact with Ca^{2+} and calmodulin, which act as secondary messengers for regulating different cellular processes in plants. H_2O_2 also interacts with salicylic acid, nitric oxide, abscisic acid, ethylene and jasmonates (Quan et al. 2008). Superoxides and H_2O_2 may result in the slow formation of hydroxyl radical ($^{\cdot}OH$) which are the most reactive of all ROS forms and initiate radical chain reactions responsible for the irreversible modification

of cellular macromolecules and damage to cell organelles. Plant cells are unable to scavenge this form of ROS (·OH) and its production leads to PCD (Karuppanapandian et al. 2011). ROS can be generated in mitochondria, chloroplasts, peroxisomes, endoplasmic reticulum and plasma membranes.

Cells have evolved a variety of enzymatic mechanisms for scavenging ROS of which superoxide dismutases (SODs; EC 1.15.1.1)—a group of metalloenzymes, form the first line of defence against ROS in plant tissues (Apel and Hirt 2004). They cause the breakdown of superoxide anion (O_2^-). Peroxidases (PODs; EC 1.11.7), another category of ROS scavenging enzymes, are heme-containing glycoproteins and include ascorbate peroxidase, cytochrome *c* peroxidase and catalase peroxidase. Peroxidases are also secreted into the cell wall or the surrounding medium and vacuole (Cosio and Dunand 2009) and are known to play a role in oxidative stress response, lignification, suberization, cross-linking of cell wall components, auxin metabolism and salt tolerance (McInnis et al. 2006b). Accumulation of ROS has been reported earlier in the stigma (receptive stage) of *Senecio squalidus* (Asteraceae) and *Arabidopsis thaliana*, suggesting their roles during pollen–stigma interaction (McInnis et al. 2006a). In the stigmas of *Senecio squalidus*, five peroxidase isoforms have been localized, out of which one is a stigma-specific peroxidase (SSP). This suggests that SSP has a unique role in the functioning of stigma. Nitric oxide (NO), a gaseous signalling molecule in plant and animal cells, reacts with oxygen in an aqueous environment, leading to the formation of nitrite and nitrate ions. NO can also react with superoxide anion, producing peroxynitrite which causes chemical modifications of proteins, including nitrosylation, nitration and oxidation of proteins (Courtois et al. 2008; Neill et al. 2003). NO has been reported to modulate floral induction and it has been reported to accumulate in the pollen grains and stigmatic papillae of *Arabidopsis* and olive (Seligman et al. 2008). The stigmatic exudates in olive also display an accumulation of NO (Zafra et al. 2010).

Sunflower (*Helianthus annuus* L.) is an important oilseed crop belonging to family Asteraceae where the inflorescence is a capitulum, consisting of numerous florets crowded together in a spiral pattern. Present investigations are aimed at monitoring the changes in the production of ROS and NO accompanying the attainment of stigma maturity in sunflower. Subsequently, experiments have been taken up to investigate the scavenging of ROS through SOD and POD activities. In this context, qualitative and quantitative analysis of SOD and POD activities have led to interesting findings on their regulatory roles accompanying stigma maturation for pollen recognition. In the present work, NO has been localized by using a novel, NO-specific probe; MNIP-Cu{(4-methoxy-2-(1H-napthol [2,3-*d*] imidazol-2-yl) phenol)-copper}, was synthesised in the author's laboratory for the fluorescence localization of NO by confocal microscopy (Yadav et al. 2013). Last, the possible role/s of ROS and NO during stigma maturation and during early stages of pollen-stigma interaction has also been investigated.

Materials and methods

Plant material

Seeds of sunflower (*Helianthus annuus* L. cv. Morden) were obtained from the University of Agricultural Sciences, Bangalore (India), washed and imbibed in distilled water for 4 h and were sown in the garden at the Department of Botany, University of Delhi. Plants were raised to reach the reproductive stage and the flowers were excised at anthesis (stage 5.3 according to Schneiter and Miller 1981), when 30 % of the head area was in flowering stage. Three morphologically different stages of floret maturation were identified (bud, staminate and pistillate). Florets at the staminate stage were excised from the capitula and their anther caps were removed. Pollen grains adhering to the outer non-receptive stigma surface were blown off with help of a brush. Florets with unopened stigmas were implanted in Petri dishes containing 1 % agar (boiled and jelled), 10 % sucrose and 100 ppm boron. The Petri dishes were covered and incubated at room temperature for 24 h. During this period, the pistils attained the pistillate stage thereby opening and revealing the receptive papillae which were then pollinated with self-or cross-pollen grains.

In situ localization of reactive oxygen species (ROS)

ROS were localized using 2,7 dichlorodihydrofluorescein diacetate as a fluorescent probe (McInnis et al. 2006a). Stigmas of specified stages were treated for 10 min with 50 μM 2,7 dichlorodihydrofluorescein diacetate (DCFH-DA) prepared in MES-KCl buffer (5 μM KCl, 10 mM MES, 50 μM $CaCl_2$, pH 6.15) at 25 °C, followed by a washing in fresh buffer for 15 min. Some stigmas were treated for 30 min with 4 mM ascorbic acid (scavenger of ROS) prepared in MES-KCl buffer (pH 6.15), followed by incubation in 50 μM DCFH-DA. Stigmas incubated in MES-KCl (pH 6.15) buffer served as controls. ROS-producing sites were observed as green fluorescing entities (ex. 485 nm; em. 515 nm) by confocal laser scanning microscopy (Model: Pascal from Zeiss, Germany) and fluorescence was quantified using Axiovision software (Zeiss, Germany).

Quantification of reactive oxygen species (ROS) in stigma homogenates

Five-hundred milligram of each tissue was powdered in liquid nitrogen and homogenized in the grinding medium [0.1 M Tris (pH 7.5), 0.4 M sucrose, 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA] containing 1 mM PMSF (phenylmethylsulfonyl fluoride), using the methods of Manna et al. (2011). Tissue homogenates were filtered through four layers of muslin cloth and centrifuged at 10,000×*g* for 20 min at 4 °C. ROS was estimated using de-esterified 2,7-dichlorofluorescein (DCFH) obtained from DCFH-DA by hydrolysis in 0.01 N NaOH (Smirnova et al. 2009; Yadav and Bhatla 2011). The aliquots of supernatants obtained from stigma homogenates (equivalent to 100 µg protein) were incubated with 5 µM of the probe (de-esterified DCFH) for 20 min at 4 °C, followed by measurement of fluorescence. Presence of ROS enhanced the fluorescence intensity of de-esterified DCFH. Ascorbic acid used in different concentrations (0.25–2 mM) led to a decrease in fluorescence intensity of the stigma homogenates. Fluorescence produced was quantified using multimode fluorescence reader (Model: LB 941, Berthold Technologies, Germany) set at an excitation wavelength of 485 nm. Emission was measured at 535 nm.

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

Synthesis of MNIP-Cu{(4-methoxy-2-(1H-napthol [2,3-*d*] imidazol-2-yl) phenol)-copper} was undertaken in the author's laboratory, using the methods of Ouyang et al. (2008), with minor modifications (Yadav et al. 2013). Use of diaminofluorescein (DAF) as a probe to localize NO has certain constraints since it does not directly bind with NO. It rather binds with N_2O_3 which can be produced from more than one biochemical route in plant tissues (Planchet and Kaiser 2006). MNIP thus synthesised was characterized by ^{1}H NMR (JEOL, Delta Spectrometer, Japan) and IR spectroscopy. Synthesis of cupper derivative of MNIP (MNIP-Cu) was always undertaken fresh just before use (Yadav et al. 2013). Dilution of MNIP-Cu used in the present work (50 µM) was obtained from a primary stock of 10 mM.

In situ localization of nitric oxide (NO)

Nitric oxide was localized on the stigma surface by confocal laser scanning microscopy (CSLM), using MNIP-Cu, a NO-specific fluorescent probe (Ouyang et al. 2008). Stigmas of specified stages were immersed in 1.0 mL of 50 µM of MNIP-Cu in Tris buffer (pH 7.4) at 25 °C for 10 min, followed by washing in fresh buffer for 10 min. Some stigmas were also treated for 60 min with 1 mM PTIO (2-phenyl-4,4,5,5 tetramethylimidazoline-1-oxyl-3-oxide), a well known scavenger for NO (prepared in Tris buffer, pH 7.4), followed by an incubation of the tissue in 50 µM of MNIP-Cu. Stigmas incubated in Tris (pH 7.4) buffer served as controls. CLSM analysis of stigmas was undertaken to identify the fluorescence indicative presence of NO (ex. 365 nm and em. 460 nm) using Argon-UV lasers and a pin hole of one (Leica, Model: TCS SP 2, Germany). The fluorescence was also quantified using Axiovision software (Zeiss, Germany).

Estimation of superoxide dismutase (SOD) activity

SOD activity was estimated spectrophotometrically,as described by Beauchamp and Fridovich (1971). The procedure for the extraction and activity estimation remained the same as for zymographic detection of SOD. Fifity microgram of TSP (total soluble protein), each from the specified stages of developing stigma, was incubated at 27 °C with 3 mL of substrate solution (9.9 mM L-methionine, 1.67×10^{-4} M nitro blue tetrazolium, 2.4×10^{-6} µM riboflavin 0.025 % Triton × 100 in 50 mM phosphate buffer, pH 7.8). Reaction was started by placing the tubes (containing reaction mixture) near a 60-W tungsten bulb (at a distance of 20 cm) for 10 min. Blank and controls were run in the same manner but in the absence of illumination and enzyme, respectively. The photochemical reaction was stopped by covering the tubes with a black cloth and absorbance was recorded at 560 nm. One unit of SOD activity refers to the amount of enzyme required for 50 % inhibition of NBT reduction in the specified assay conditions and its activity was expressed in units mg^{-1} protein. The activity of different isoforms of SOD was detected using the method of Prochazkova et al. (2001) whereby KCN (3 mM) was used to inhibit Cu/Zn-SOD isoforms and KCN (3 mM) and H_2O_2 (5 mM) together were used to inhibit both Cu/Zn-SOD and Fe-SOD activities.

Zymographic detection of superoxide dismutase (SOD)

Superoxide dismutase (EC 1.5.1.1) isoforms were detected zymographically (Beauchamp and Fridovich 1971; Shine et al. 2012). 500 mg stigmas from each developmental stage were collected, powdered in liquid nitrogen and homogenized in 1.5 mL of Tris–HCl buffer (50 mM, pH 7.0) containing 50 mM NaCl, 0.05 % Tween-80 and 1 mM PMSF. The homogenates were centrifuged at 10,000*g* for 20 min and the supernatants obtained after centrifugation were used for estimating SOD activity. 50 µg of TSP from each homogenate was mixed with non-reducing Laemmli sample buffer and loaded on 15–20 % gradient gel.

Vertical gel electrophoresis was performed at 25 mA for 5 h at 4 °C and the gel was subsequently soaked in 2.5 mM nitro blue tetrazolium (NBT) for 25 min. The gel was then incubated for 20 min in dark, in 50 mM phosphate buffer (pH 7.8) containing 28 μM riboflavin and 28 mM TEMED, transferred to distilled water and exposed to light through a light box for 10–15 min at room temperature. During illumination the gel became uniformly blue expect at positions containing SOD activity. Illumination was stopped when maximum contrast between achromatic zone and blue colour was achieved.

Estimation of peroxidase (POD) activity

Total peroxidase activity was estimated spectrophotometrically (Alba et al. 1998). Enzyme assay was performed by mixing 50 μg protein (as enzyme source from different stages of stigma) with 2.4 ml of substrate solution (0.6 mM o-dianisidine and 8.8 mM H_2O_2 in 50 mM sodium phosphate buffer, pH 6.0). The procedure for the extraction of total protein from the tissue homogenates for POD activity estimation was the same as for zymographic detection of POD. Absorbance change was recorded at 460 nm up to 8 min against a blank containing 2.4 mL of substrate mixed with sodium acetate buffer (pH 6.0). The extinction coefficient of dianisidine oxidized product is 11.3 mM^{-1} cm^{-1} and enzyme activity was expressed as specific activity of POD in mol $\mu g^{-1} min^{-1}$.

Zymographic detection of peroxidase (POD) activity

Peroxidase isoforms were detected zymographically, using the methods of Alba et al. (1998). Homogenates were prepared by grinding the tissue in 50 mM sodium acetate buffer (pH 4) and filtered through four layers of muslin cloth. The filtrate was centrifuged at 10,000*g* for 20 min at 4 °C and was subjected to acetone precipitation of proteins by mixing supernatant with four volumes of acetone, followed by storage of the vials for approximately 12 h at −20 °C. The precipitated protein was dissolved in 400 μL of 0.1 M Tris (pH 7.5). Aliquots of TSP equivalent to 100 μg protein were mixed with reducing Laemmli sample buffer and loaded on 15–20 % gradient gel. Vertical gel electrophoresis was performed at 25 mA for 5 h at 7 °C. After electrophoresis, the gel was incubated for 20–30 min in 0.2 M sodium acetate buffer (pH 5.0) containing 1.3 mM benzidine (24 mg in 100 ml) and 1.3 mM H_2O_2 (4 μL in 100 ml) until brown bands representing peroxidase isoforms appeared.

Wherever required, protein was quantified using the methods of Bradford (1976). All biochemical experiments were performed at least thrice with at least three technical replicates in each experiment. In situ localization of NO and ROS was performed in at least 8–10 samples for each treatment.

Results

Development of inflorescence in sunflower

The capitulum of sunflower possesses disc (inner) and ray (outer) florets. The ray florets are incomplete sterile and possess with a yellow petal while the disc florets are complete with bracts, bracteole, stamen and pistil. The disc florets at anthesis (stage 5.3, according to Schneiter and Miller 1981) can be distinguished into three different developmental stages, namely bud, staminate and pistillate stage. Young buds are present in the centre of the capitulum, and as the bud florets mature the anthers in the florets turn brown. Simultaneously, the height of the florets increases along with an enlargement of the ovary (Fig. 1a). Sunflower exhibits protandry during floral development which elongates the floret life, thereby allowing more pollinators to visit the capitulum. The syngenesious stamens are exposed through the tubular corolla at the staminate stage and, thereafter, pollen grains are released. The style elongates through the anthers, exposing the pollen grains lying inside the anther lobe. As the style elongates, it detaches medially, curling outward and exposing the inner receptive surface at the pistillate stage of the floret.

Papillae accumulate reactive oxygen species (ROS) with the maturation of stigma

Treatment of stigma with the fluorescent probe (DCFH-DA) at the specified stages of development (bud, staminate and pistillate) and subsequent confocal microscopic image analysis has revealed that with the progress of stigma development the intensity of green fluorescence due to ROS accumulation in the cells of papillae also increased. This fluorescence was largely neutralized when the probe-treated stigmas were co-incubated with ascorbic acid, an ROS scavenger (Fig. 1b). The intensity of relative fluorescence units due to ROS also increased at the pistillate stage in comparison with bud and staminate stages of stigma development (Fig. 1d). With the advancement of stigma development, there was also a gradual and significant accumulation of ROS in the whole tissue homogenates (Fig. 2a). Co-addition of increasing concentrations of ascorbic acid (0.25–2 mM) to the tissue homogenate led to a decrease in fluorescence due to ROS, indicating that ascorbate peroxidase activity (which is responsible for scavenging the accumulating ROS) is prevalent in the stigma tissue (Fig. 2b). In addition to acting as substrate for ascorbate peroxidase, ascorbic acid can also non-enzymatically donate electrons, leading to scavenging of $O_2^{\cdot-}$, $OH^{\cdot}$ and $^1O_2^{\cdot-}$.

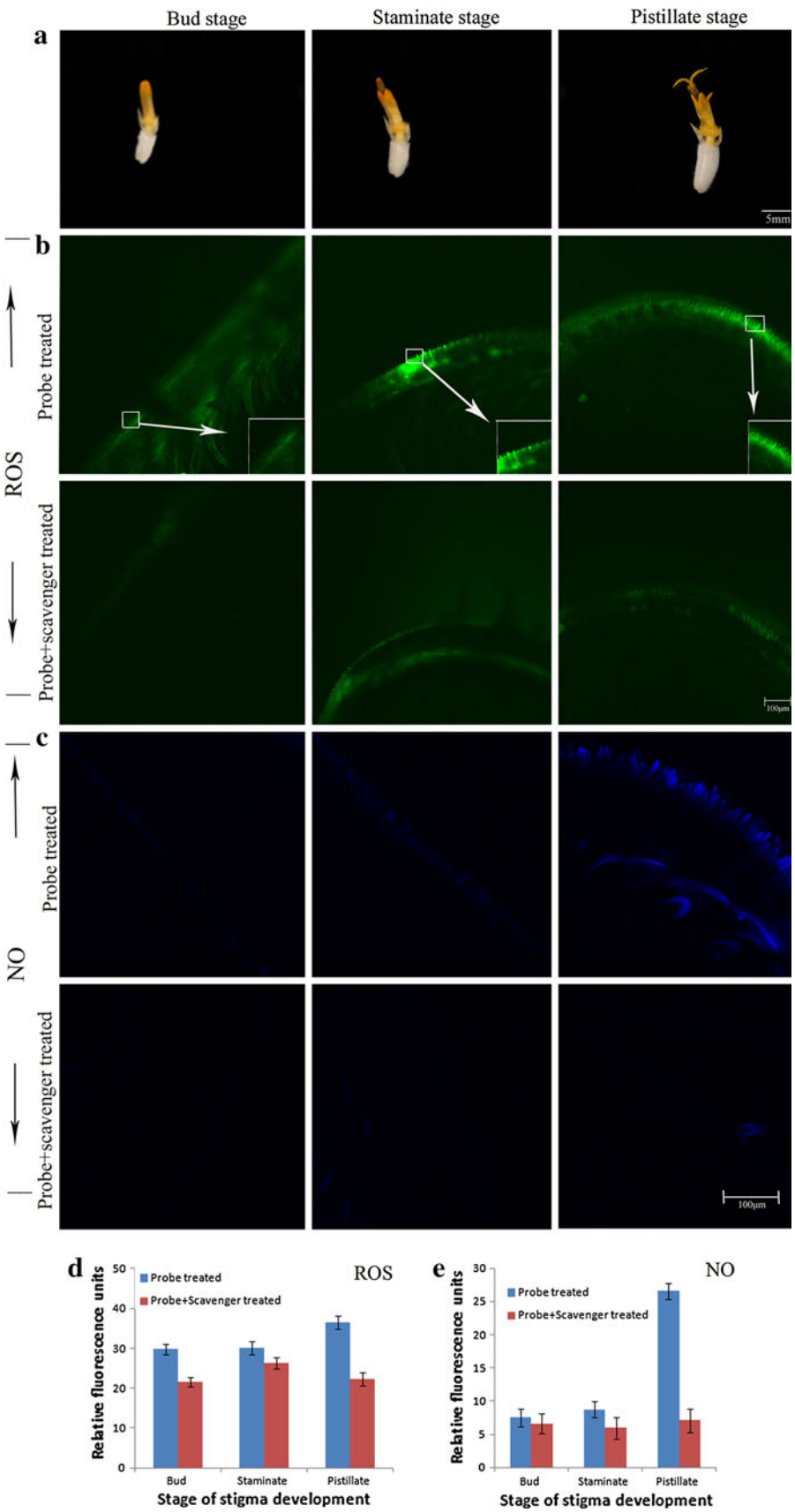

Fig. 1 Floret development in a capitulum of sunflower. **a** Bud stage, staminate stage, pistillate stage. **b** Localization of ROS at different stages of stigma development by treatment with DCFH-DA. Scavenger used: Ascorbic acid (4 mM). Magnification: ×100. **c** Localization of NO at different stages of stigma development by treatment with MNIP-Cu. Scavenger used: PTIO (1 mM). All images represent consolidation of observations from stacks obtained during confocal image analysis. Magnification: ×200. *Bar* represents 100 μm. **d** Quantification of fluorescence due to ROS as observed in **b**. **e** Quantification of fluorescence due to NO as observed in **c**. Quantification data represent mean values and standard errors from at least ten replicates

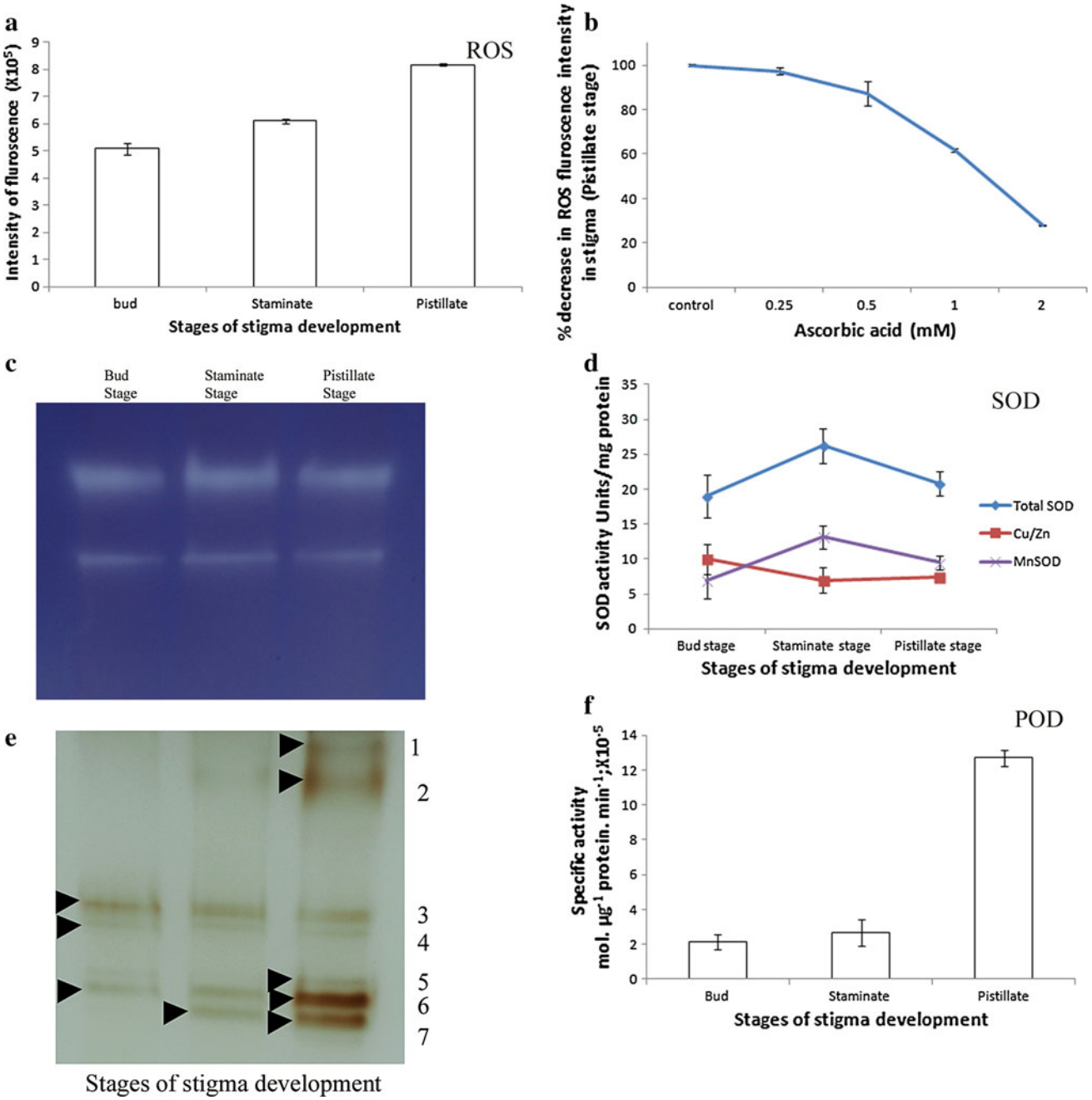

Fig. 2 Spectrophotometric analysis of ROS accumulation and the activity of ROS scavenging enzymes. **a** ROS activity in tissue homogenates at three stages of stigma development. **b** Effect of ascorbic acid treatment on ROS. **c** Zymographic detection of SOD isoforms following treatment with nitro blue tetrazolium (NBT). **d** Changes in specific activity of total superoxide dismutase (SOD) activity, Cu/Zn and Mn-SOD in the cytosolic fraction from stigma homogenates from the three stages of development. **e** Zymographic detection of POD isoforms following benzidine treatment. **f** Changes in specific activity of total POD in the cytosolic fraction from stigma homogenates from the three stages of development. Quantitative data represent mean and standard errors from three replicates

ROS scavenging is facilitated by an increase in superoxide dismutase (SOD) activity in the developing stigma

SOD catalyses the dismutation of superoxide radicals to H_2O_2, which is followed by the scavenging of H_2O_2 by peroxidases. Zymographic detection of SOD isoforms at the three stages of stigma development revealed two constitutive SOD isoforms (Fig. 2c). Total SOD activity in the sigma tissue homogenates (10,000*g* supernatant) was low at the bud stage, got elevated at the staminate stage and reduced again at pistillate stage of stigma development.

Differential pattern of enzyme activity was evident for Mn-SOD (localized in mitochondria) and Cu/Zn SOD (localized in peroxisomes and cytoplasm) at the three stages of stigma development (Fig. 2d). Thus, Mn-SOD increased at the staminate stage of stigma development, whereas Cu/Zn-SOD was high at the bud stage and its activity was reduced at the staminate and subsequent stages of stigma development.

ROS scavenging in the receptive stigma is achieved by a high peroxidase (POD) activity through the expression of new isoforms

Zymographic analysis of POD isoforms revealed only three POD isoforms at the bud stage, four at the staminate stage and seven at the pistillate stage of stigma (Fig. 2e). Furthermore, a differential expression of the POD isoforms at the pistillate stage revealed that POD accumulation at this stage was primarily due to enhanced expression of two isoforms (number 6 and 7). Specific activity of POD remained at its minimal and at similar level during the bud and staminate stages of developing stigma (Fig. 2f). It exhibited fivefold increase at the mature (pistillate) stage of development compared with younger stages. It is, thus, evident that the observed increase in POD activity accompanying stigma maturation is due to qualitative and quantitative changes in the activity of POD isoforms.

Attainment of stigma receptivity accompanies nitric oxide (NO) accumulation in the papillae

NO has been localized by subjecting whole stigmas with an NO-specific fluorescent probe (MNIP-Cu), which is known to possess NO-specific binding affinity (Ouyang et al. 2008). MNIP-Cu overcomes the disadvantages associated with DAF (as stated under "Materials and methods"; Yadav et al. 2013). Application of MNIP-Cu has earlier been established for in vitro and in vivo detection of nitric oxide produced by inducible nitric oxide synthase in animal cells (Ouyang et al. 2008). Keeping in view its permeability in animal cells, this probe has been successfully used by us recently to monitor endogenous NO localization in the hypocotyl explants and protoplasts derived from dark-grown sunflower seedling (Yadav et al. 2013). Furthermore, symplastic and protoplastic blue fluorescence, which is evident in the cells of the papillae, has also been quantified in the present work (Fig. 1e) using Axiovision software (Zeiss, Germany). Thus, the present work highlights the use of MNIP-Cu for qualitative and quantitative fluorescence detection of nitric oxide in stigma papillae. Treatment with this probe showed intense blue fluorescence in the stigma papillae at the pistillate stage of development (Fig. 1c). The intensity of relative fluorescence units due to NO was lesser at the younger stages of stigma development than the pistillate stage, which was quenched upon treatment PTIO, a well-known NO scavenger (Fig. 1e).

An inverse correlation between ROS and NO accumulation is evident in pollen and stigma during pollination

In the present investigation, pollinated stigmas exhibited a decrease in ROS accumulation at the sites where pollen grains were adhering to the papillae (Fig. 3a, b). Negligible fluorescence was also observed in the pollen grains, indicating that pollen grains may have a small amount of ROS accumulation (Fig. 3a, b inset). The papillae in both self- and cross-pollinated conditions showed similar levels of ROS accumulation. The intensity of fluorescence due to NO was more in pollen grains as compared with the stigmatic papillae on the pollinated stigmas (Fig. 3a, b). NO was localized in the exine region of pollen grains and in the apoplastic region of the tips of papillae. Upon cross pollination, germinating pollen grains revealed the presence of NO in the exine region and the pollen tube wall (Fig. 3a). The adjoining papillary cells also showed some accumulation of NO at the apical regions. Pollen grains did not germinate after self-pollination and an intense NO accumulation was detected on its surface (Fig. 3b).

Discussion

Floral development among the members of Asteraceae is governed by signals, like photoperiod (Baroncelli et al. 1990) and plant hormones, such as auxins, gibberellins and ethylene (Koning 1983). The elongation of staminal filament is controlled by phytochrome at the staminate stage of floret development (Baroncelli et al. 1990). The style lengthens through the anther tube, pushing the pollen grains out and then detaches medially, with the tips curling outward (Sammataro et al. 1985). Members of Asteraceae show the phenomena of secondary pollen presentation, whereby pollen grains are relocated from anthers to other floral organs, which then present the pollen for pollination (Howell et al. 1993). As the style elongates, pollen grains are released and those pollen grains left in the anther tube adhere to the pseudopapillae and are exposed to the pollinators (Hong et al. 2008). Recently, there have been reports on the accumulation of ROS/H_2O_2 in the stigmatic papillae of angiosperms, which may be involved in the signalling events leading to pollen germination and tube growth (McInnis et al. 2006a; Hiscock et al. 2007; Zafra et al. 2010). In the present investigation, accumulation of ROS increases in the stigmatic papillae as the stigma attains receptivity. Ascorbic acid, a scavenger of H_2O_2, is

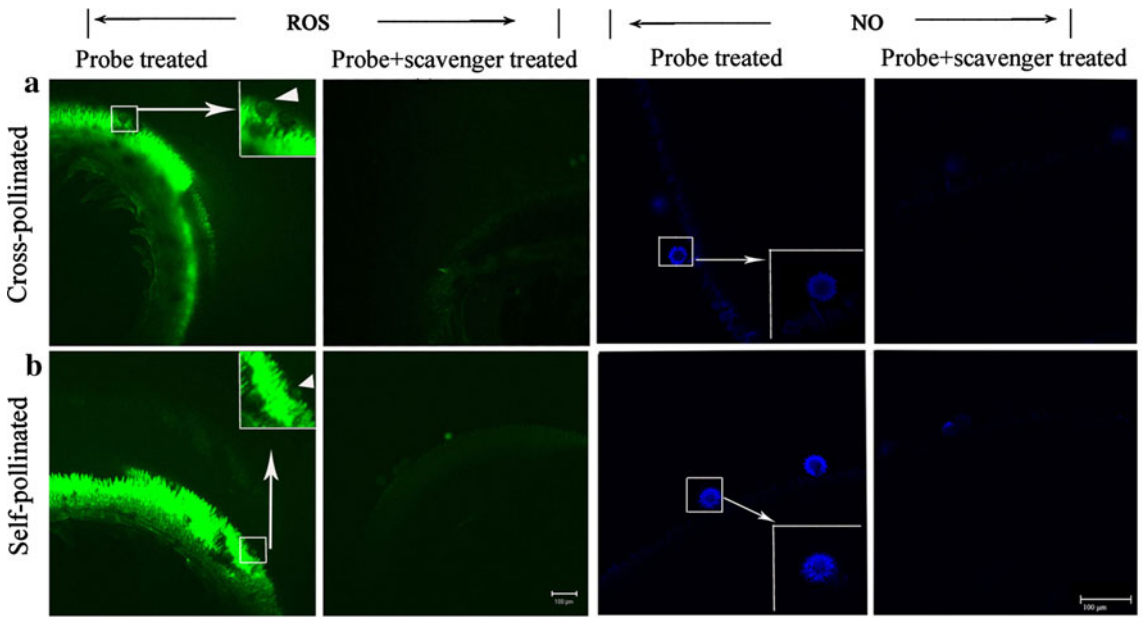

Fig. 3 Changes in the accumulation of reactive oxygen species (ROS) and nitric oxide (NO) in pollen and stigma in relation with self- and cross-pollination. **a** Localization of ROS and NO at the pistillate stage of stigma, after cross-pollination. Magnification: ×100. **b** Localization of ROS and NO in pistillate stage of stigma after self-pollination. Magnification: ×200. All images represent consolidation of observations from stacks obtained during confocal analysis

able to quench fluorescence due to ROS (Barceló 1998) obtained by treating stigmas with DCFHDA. Thus, fluorescence due to ROS in sunflower stigma is mainly due to H_2O_2 accumulation, although scavenging of other ROS is also not ruled out (Karuppanapandian et al. 2011). Quantification of ROS in the stigma tissue homogenates (10,000*g* supernatant) further supports the observations made by confocal microscopic image analysis. Presence of high ROS/H_2O_2 and stigma-specific peroxidase activity can lead to cross-linking of pectins and extensins in the cell wall (Passardi et al. 2004), resulting in pollen adhesion and pollen tube growth in the stigma. In contrast to the present results, attainment of stigma receptivity accompanies a decrease in ROS accumulation in olive (Zaffra et al. 2010). This is proposed to be due to the adherence of pollen grains on the receptive stigma (Serrano et al. 2012b). Stigma is also a source of nutrients for pollen grains and is prone to microbial attack. High levels of ROS have earlier been detected in the floral nectar, indicating their role in defence against pathogens (Carter and Thornburg 2004). ROS may directly be toxic against pathogenic microorganisms or may trigger hypersensitive reactions and PCD at the sites of pathogen attack (De Rafael et al. 2001).

Superoxide dismutases (SODs) form the first line of defence against ROS accumulation in the vegetative and reproductive plant tissues by causing a breakdown of superoxide anion (O_2^-). SODs generate H_2O_2 which contributes to antimicrobial activity in the nectar (Carter and Thornburg 2004). In *Nicotiana tabacum*, five proteins have been reported to accumulate in the nectar which function in redox cycle, leading to the production of high levels of H_2O_2 in the nectar. The expression of NEC I (Nectarin I) is associated with Mn-SODs activity (Carter and Thornburg 2004; Carter et al. 2007). In the present work on sunflower, the activity of Mn-SOD increases during the staminate stage of stigma development, whereas the activity of Cu/Zn SOD decreases (Fig. 2d). This indicates high mitochondrial Mn-SOD activity due to enhanced nectar secretion or increased mitochondrial activity accompanying rapid elongation of stigma and anther dehiscence at the staminate stage of stigma development. In sunflower, nectar is secreted at the base of the florets during the pollen producing stage of flowering and also while stigma is receptive (Tripathi and Singh 2008). In Petunia, SOD activity has been reported to increase during the anther dehiscent stage (Wang 2006). A reduced SOD activity at the pistillate stage of stigma development in sunflower (present work) indicates accumulation of this form (O_2^-) of ROS in mature stigma. Distribution of superoxide anion has also been reported to increase during the receptive stage of stigma development in olive (Zafra et al. 2010; Serrano et al. 2012b). In olive, superoxide anion accumulation increases after pollination. It has been proposed that superoxides in association with NO from the pollen grains lead to the formation of peroxynitrite, which causes PCD of papillar cells and self-incompatible pollen grains (Serrano et al. 2012a).

Peroxidase (POD) isoforms form a component of signalling network, mediating species-specific pollen recognition (McInnis et al. 2005). In *Arabidopsis thaliana*, *Petunia hybrida* and *Senecio squalidus* POD activity increases as stigma matures, suggesting its importance in the attainment of stigma receptivity (Dafni and Maués 1998; McInnis et al. 2006b). Peroxidases are associated with the thin pellicle which overlays the cuticle in dry and semi-dry stigmas, and it is the first layer that comes in contact with pollen grains during pollen–stigma interaction (McInnis et al. 2006b). Similar to the present observations on sunflower, low POD activity has earlier been detected in the developing stigmas of *Peduclaris canadensis* and *Clintonia borealis* (Galen and Plowright 1987). The present work also reports the expression of specific POD isoforms during stigma receptivity indicating their specialized roles (Fig. 2e). Three peroxidase isoforms are specific to stigma in *Arabidopsis.* One peroxidase isoform has been identified as stigma specific in *Senecio squalidus,* and one putative stigma specific peroxidase has been identified in hazelnut (Beltramo et al. 2012). Pistil expresses a number of pathogenesis-related proteins, some of which are peroxidases. A peroxidase isoform in *Capsicum annuum* L. shows increased expression as a response to pathogen (Beltramo et al. 2012). In the style of *Nicotiana* sp., an increased activity as well as induction of new POD isozymes has been observed upon ageing (Bredemeijer 1984). The increased POD activity at the pistillate stage of stigma development indicates its diverse role/s accompanying the attainment of stigma receptivity, recognition of species-specific pollen, their germination and penetration of pollen tube in the stigmatic tissue. POD isoforms may also maintain a fine balance among the various ROS forms essential for different developmental processes. Zygmographic detection of new POD isoforms at the pistillate stage of development may also be an indication of senescence. An interaction between NO in the ungerminated pollen and ROS in stigma has earlier been proposed in *Senecio squalidus* and *Arabidopsis thaliana* (Hiscock et al. 2007; McInnis et al. 2006a). It has been proposed that NO might serve as a substrate for some isoforms of mammalian peroxidases (Abu-Soud and Hazen 2000). Peroxidases may function as a catalytic sink for NO, thereby influencing its (NO) bioavailability. Further work on these lines is likely to elucidate the mechanism of interaction between NO and peroxidase.

The present investigations have shown accumulation of NO at the pistillate stage of stigma development. Similar observations have earlier been reported in the developing stigma of olive (Zafra et al. 2010) and *Arabidopsis* (Seligman et al. 2008). NO production in *Arabidopsis thaliana* coincides with the maturation of pollen and papillae before anthesis (Seligman et al. 2008). Nitric oxide is known to provide immunity in plants and, thus, plays an important role in defence mechanisms (Asai et al. 2010). It has been proposed that NO can act as a protectant against heat stress, by activating ROS scavenging enzymes, thereby causing thermotolerance (Piterková et al. 2013). The presence of NO at the advanced stages of stigma development in olive has been reported to indicate senescence (Zafra et al. 2010). In olive, NO is not detected before pollination, and both NO and $O_2^{\cdot-}$ coexist after pollination and cause PCD after pollination (Serrano et al. 2012a). In the present investigation, ROS and NO accumulation show an inverse correlation in the stigmatic papillae and ungerminated pollen (Fig. 3). NO seems to reduce the ROS/H_2O_2 accumulation in the stigmatic papillae at the point of its contact with the pollen grains. The present investigation in sunflower does not show a difference in ROS accumulation in self- or cross- pollinated conditions (Fig. 3a, b). In olive, H_2O_2 accumulation in the stigmatic papillae decreases upon pollination. The decrease in H_2O_2 accumulation is similar both in self- or cross-pollinated situations (Serrano et al. 2012b). The present work shows that upon self-pollination, sunflower pollen grains do not germinate and high levels of NO is detected on the surface of pollen grains. Compatible pollination results in hydration and germination of pollen grains. The pollen tube grows towards the basal region of the stigmatic papillae. NO accumulation has been detected in the walls of pollen tubes and in the apical region of the papillae. NO has been detected in the pollen grains before germination in a variety of plants (Bright et al. 2009; Wilson et al. 2009), pollen apertures and in the pollen tubes in olive (Zafra et al. 2010). It is absent in the pollen tip region and is detectable at higher levels in the subapical regions of *Lilium longiflorum* (Prado et al. 2004; Speranza et al. 2012). NO production has earlier been correlated as a negative regulator with the rate of pollen tube growth (Zafra et al. 2010). Thus, ROS–NO interaction seems to operate between stigma and pollen, triggering compatible or incompatible response.

To sum up, present investigations have revealed an accumulation of ROS/H_2O_2 with the maturation of stigma. SOD and POD activity increase, indicating their role in balancing the levels of ROS/H_2O_2. The increase in SOD activity is mainly due to Mn-SODs which leads to the formation of peroxides. POD is likely to cause the breakdown of excess ROS/H_2O_2 in the stigma at the pistillate stage. NO is absent in the young stages of stigma development and it accumulates as stigma matures. NO may be involved in the later events related to senescence. An interaction between NO and ROS is likely to modulate the event of pollination. Accumulation of ROS and NO is inversely related in stigma and pollen grains, respectively. NO and ROS are likely to interact in the events of

recognition between the stigma and pollen. NO/ROS may be involved in PCD causing an arrest in the development of self pollen. Compatible pollination results in the formation of a pollen tube which shows NO accumulation in the walls of pollen tubes. Further investigations to elucidate the exact nature of interaction between NO and ROS in pollen tube and papillae hold promise for an understanding of the mechanisms of pollen-stigma interaction.

Author contribution Basudha Sharma has contributed by performing most of the experiments in the laboratory. SC Bhatla has contributed in the form of compilation of data, literature survey, data analysis and preparation of the manuscript. Basudha Sharma is working for her Ph. D. Degree under SC Bhatla and the work submitted in the present manuscript is a part of her research work.

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