Preharvest application of hydrogen sulfide and nitric oxide improves floral traits and postharvest performance of cut gladiolus inflorescences

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Abstract

Gladiolus is a well-known bulbous plant producing impressive cut spikes. Hydrogen sulfide and nitric oxide are vital signaling molecules for the proper functioning of plant metabolism. Preharvest applications of these molecules to crops have gained attention in recent years due to their positive role in tackling abiotic stresses, although, their role in geophytes is comparatively less studied. We assessed the effects of preharvest H_2S and NO treatments on development, flowering, harvest and postharvest performance of gladiolus inflorescences. NO and H_2S+NO treatments increased preharvest performance of plants associated with corm production, inflorescences length and harvest time. Individual and combined treatments improved postharvest vase life (VL) up to 3.4 d. Total soluble proteins (TSP) were increased in response to H_2S, NO and H_2S+NO treatments by 39%, 43%, and 55%, respectively compared to the controls. Soluble sugars (SS) were increased after NO and H_2S+NO treatments by up to 25% and 42%, respectively. Postharvest catalase (CAT) activity was higher by 65%, 68%, and 76% after H_2S, NO and H_2S+NO treatments, respectively. Malondialdehyde (MDA) was decreased by all preharvest treatments by up to 88%, although, only the combined H_2S+NO treatment reduced H_2O_2 and superoxide dismutase (SOD) activity. The results confirm that preharvest treatments with H_2S, NO and H_2S+NO may positively affect growth, floral traits and postharvest performance of cut gladiolus inflorescences.

Keywords: Soluble sugars; soluble proteins; antioxidants; hydrogen peroxide, malondialdehyde; vase life; leaf gas exchange

1. Introduction

Gladiolus grandiflorus L. (Iridaceae), a perennial herbaceous, monocotyledonous, geophyte is an economically important ornamental plant. It is commonly grown to produce cut flowers, and for the beautification purpose in the landscape garden as potted or landscape ornamental plant. Cut flowering spikes of gladiolus are highly ranked commercially in the international cut flower markets, with constant demand all year round (Zulfiqar et al., 2023). According to recent sales statistics, there exists continuous demand for cut gladiolus inflorescence for local markets and for exports to specific countries. A successful business of cut flower production demands short and predictable production time, minimum production cost, and the postharvest vase life (VL) for sale in distant markets.

VL is one of the vital quality factors that influence the marketability and customers' satisfaction in buying cut gladiolus flowers. Senescence is the major factor for reduced VL and quality loss after harvest (Shabanian et al., 2018). Senescence is associated with ultrastructural changes, increases in membrane leakage and lipid peroxidation, increased hydrolytic enzyme activities, enhanced respiration rates, macromolecule degradation and modifications in different cell organelles (Mansouri, 2012; Rani and Singh, 2014). Furthermore, various other factors stimulate the initiation of the senescence process (Reid and Jiang, 2012), such as preharvest considerations such as the genotype, the cultivation process, and the environmental factors (e.g. humidity, light, water relations, temperature, and nutritional status) (Fanourakis et al., 2013). At the postharvest phase, factors such as ethylene sensitivity, vase solution microbial activities, and oxidative stress may also affect quality and senescence (Rani and Singh, 2014). Hence, the delay of senescence is vital to attain high commercial values (Gong et al., 2018). In view to maximise VL, researchers continuously search for new and effective postharvest strategies that delay the senescence process.

Hydrogen sulfide (H_2S) may act as a signaling molecule to alleviate abiotic stress, delay senescence and improve postharvest quality of horticultural crops (Zulfiqar and Hancock, 2020). Exogenous H_2S treatments improved growth and postharvest performance of horticultural produce by increasing the endogenous accumulation of H_2S, the intracellular ATP and NADPH and the activities of reactive oxygen species (ROS) scavenging enzymes. H_2S treatments maintained energy status, inhibited the chlorophyll degradation and respiration rate, and enhanced antioxidant capacity during postharvest (Hu et al., 2015). Liu et al. (2017) evaluated the involvement of H_2S during the postharvest performance of day lily and observed that H_2S contribute in enhancing VL and reducing senescence of postharvest daylilies by increasing antioxidant capacity and sustained energy status.

Similarly, nitric oxide (NO) is a highly reactive, small and diffusible endogenous gaseous signaling molecule which play vital role in plant growth and development. Leshem and Wills (1998) reported that NO acted as natural senescence-delaying agent that primarily, but not solely, down-regulated ethylene emission. Several investigations revealed that NO application can delay senescence of cut flowers (Chang-li and Guo Quan, 2011; Liao et al., 2013; Mittal et al., 2021). Dwivedi et al. (2016) stated that application of NO improved the VL of gladiolus by 2.6 d through regulation of enzymatic activity. In cut carnation flowers, NO application improved VL by decreasing ethylene production and down-regulation of ethylene biosynthesis. NO also decreased the expression of petal senescence-associated genes, and enhanced the scavenging of ROS through efficient anti-oxidation.

To date, few studies have evaluated the effects of postharvest treatments of NO to cut flowering stems. However, no studies were found on the effects of preharvest applications of H_2S and NO on plant and flower development, corm production and postharvest characteristics of

gladiolus. This study for the first time examined the regulatory effect of separate and combined H_2S+NO foliar treatments on the performance of gladiolus plants, as well as the association of photosynthetic traits with postharvest longevity and defense related enzyme activities that confer protection against postharvest oxidative stress.

2. Materials and Methods

2.1. Plant material and experimental treatments

Gladiolus corms (18-20 cm in circumference) of cv. "White Prosperity" were acquired from Sunny seeds Lahore, Pakistan which is an importer of ornamental seeds and bulbs from the Netherlands, and were grown under open field conditions during 2021 at the Floriculture research area, at IUB Pakistan. Corms treated with Topsin-M (0.02 kg L^{-1}) were planted in 2 L having top diameter 17.0 cm and pot base diameter 12.0 as well as the pot height of 13.0 cm. The trial was set up in a Completely Randomized Design, with treatments serving as the only variable. Each treatment had 15 replications, and the experiment was conducted twice underneath the same conditions.

Gladiolus plants were treated with donors of NO (0.10 mM sodium nitroprusside, SNP) or H_2S (0.20 mM sodium hydrosulfide, NaHS). The doses of both elicitors were established after preliminary tests on the field and international literature (e.g., Mittal et al., 2021). During gaseous treatments with NO and H_2S, the control plants were covered with a polyvinyl film to avoid interference with the chemical elicitors.

2.2. Vegetative and reproductive characteristics

Total leaves and average leaf area (mm^2) were evaluated using a Portable Area Meter. Total days taken to harvest were recorded. Inflorescence length (cm) and number of florets per inflorescence, were measured. On harvesting of cut gladiolus inflorescences, two base leaves were not cut with the inflorescence for assisting the underground corm development process. On maturity (yellowing and drying of leaves), corms were manually uprooted with a spade and immediately were washed under running tap water near the production site and corm mass (gr) and corm diameter (cm) were recorded.

2.3. Leaf gas exchange

At bud emergence stage of flowering, chlorophyll and leaf gas exchange (LGE) were measured. The measured parameters were performed between 8.15 am to 10.00 am, on four mature, and healthy leaves from fifteen plants of a treatment using an infrared gas analyzer set at 400 μmol m^{-2} s^{-1} CO_2 and a flow rate of 300 μmol m^{-2} s^{-1}. Chlorophyll levels were recorded using a SPAD meter on six expanded, healthy leaves.

2.4. Harvesting cut flowers and vase life evaluation

Floral spikes were manually cut at the stage of 2-3 open florets per inflorescences, using a sharp-edged knife during early morning hours. The cut inflorescences were taken to the floriculture laboratory within 20 min. While in the laboratory, the inflorescences were re-cut under water at 85 cm length to avoid blockages of the vascular system. Inflorescences were put into 200 mL glass vases (1 in each) filled with DW. All vases with inflorescences were placed at 20 ± 3 °C, relative humidity 60 ± 5 %, under 12 h light /12 h dark periods. Total fifteen cut inflorescences per treatment were used. The VL was measured as the total number of days from harvest till the day

when 60 % of the floret petals wilted or lost turgidity and/or color. Data were recorded every 2 d till the end of the VL.

2.5. Soluble sugar (SS) and total soluble protein (TSP) contents in leaves

SS content in leaves was assessed 4 d before cutting the infloresences following the methodology narrated by Frohlich and Kutscherah (1995). First, samples (0.0005 kg fresh leaves) were added to the test tubes comprising distilled water (10 mL). The sealed tubes were then incubated at 80 °C for 60 min. These tubes were then set to the volume at 25 mL. Of the collected supernatant, 0.5 mL was mixed with 0.5 mL anthrone, 1.5 mL distilled water, and 5 mL sulfuric acid. These solutions were assessed for SS (g kg^{-1}) in a spectrophotometer at 620 nm. TSP (g kg^{-1}) were assessed on a fresh weight basis in leaves following the methodology narrated by Bradford (1976).

2.6. Malondialdehyde and hydrogen peroxide contents

For measuring MDA and H_2O_2, fresh samples of 0.0005 kg of the fifth floret on the inflorescence (from base upwards) were collected on day-5 after cutting from plants. MDA and H_2O_2 contents were evaluated following Hodges et al. (1999) and Patterson et al. (1984), respectively.

2.7. Defense enzyme activities

Fresh flower samples of 0.0005 kg were taken from the fifth floret on the spike (from base upwards) on day-4 after cutting from plants. The sample was homogenized in a chilled mortar and pestle containing 5 mL of ice-cold 50 mM sodium phosphate buffer, pH 7.8, containing 2 % (w/v) polyvinylpyrrolidone and 1.0 mM EDTA. The homogenate was then centrifuged at 10,000 *g* at 4

°C for 20 min. SOD and CAT activities were determined using supernatant stored at 20 °C following the methodologies by van Rossum et al. (1997) and Chance and Maehly (1955), respectively.

2.8. Proline content

Ninhydrin-oriented method was followed to quantify leaf free-proline concentration (Bates et al. 1973). Briefly, 0.0005 kg fresh sample was extracted in 10 mL of 3 % (w/v) sulfo-salicylic acid. Then, 2.0 mL of the filtered solution was put into 2.0 mL of acid ninhydrin (1.26 g ninhydrin + 20 mL 6 M ortho-phosphoric acid + 30 mL glacial acetic acid) and 2.0 mL of glacial acetic acid. After incubation for 60 min at 80 °C, samples were immediately transferred to an ice bath for ending the reaction. Afterwards, Toluene (4.0 mL) was put into the solution and mixed vigorously by vortexing for 30 s. The chromophore comprising toluene was separated from the aqueous phase. Absorbance was recorded at 520 nm.

2.9. Statistical analysis

Data was analysed by one-way ANOVA (SPSS ver. 22; Chicago, USA) and Duncan's multiple range test ($P \leq 0.05$). Pearson correlations (2-tailed) were executed to demonstrate the relations between variables as affected by NO and H_2S treatments.

3. Results

3.1. Plant growth and corm production

177 Leaf numbers in NO- and H_2S+NO-treated plants increased by up to 12% and 17%,
178 respectively compared to the controls (Fig. 1A). Leaf area of the H_2S, NO and H_2S+NO-treated
179 plants increased by up to 6%, 5% and 9%, respectively (Fig. 1A). Harvest time was reduced
180 decreased for H_2S, NO and H_2S+NO-treated plants by 4.2, 6.6 and 11.5 d, respectively compared
181 to controls (Fig 1B). Inflorescence length increased in response to the NO and H_2S+NO treatments
182 by up to 8% and 13%, respectively (Fig 1C). Number of florets increased by 20% on the H_2S+NO-
183 treated plants (Fig 1C).
184 Corm mass increased by all treatments (Fig.2). H_2S, NO and H_2S+NO treatment increase corm
185 mass by 3, 2.5 and 6.5 gr, respectively compared to the controls (Fig 2). However, H_2S, NO and
186 H_2S+NO treatments did not affect corm diameters (Fig. 2).
187
188 *3.2. Leaf gas exchange and chlorophyll content*
189 Plants treated with H_2S and H_2S+NO showed increase in *As* by 29% and by up to 35%,
190 respectively (Fig. 3A). NO treated plants did not show any increase in *As* compared to the control.
191 H_2S+NO helped plants maintain lower transpiration, although, stomatal conductance (g_s) was not
192 affected by any of the treatments (Fig. 3B, C). The chlorophyll content (SPAD values) increased
193 under H_2S, NO and H_2S+NO treatments by up to 16%, 19%, and 18%, respectively, (Fig. 3D).
194
195 *3.3. Vase life*
196 VL was increased by all preharvest treatments. The combined H_2S + NO treatment had the
197 lengthiest VL of 13.64 d (+ 33%), followed by H_2S treatment having 12.54 d (+ 19%) and NO
198 with 11.90 d (+ 14%) as compared to the control inflorescences (10.22 d) (Fig. 4).
199

200 *3.4. Soluble sugars and total soluble protein contents*

201 SS under NO and H_2S+NO treatments were enhanced by up to 25% and 42%, respectively
202 (Fig 5A). Although, TSP were increased in response to H_2S, NO and H_2S+NO treatments by 39%,
203 43%, and 55% compared to the controls (Fig 5B).

204

205 *3.5. Malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) contents*

206 In our experiment, MDA was declined by all preharvest treatments (Fig 6A). H_2S, NO and
207 H_2S+NO reduced MDA by up to 16%, 18% and 88%, respectively. The H_2O_2 content was reduced
208 by up to 55%, only by the combined H_2S+NO treatments (Fig 6B). NO and H_2S did not
209 significantly affect H_2O_2 content.

210

211 *3.6. Superoxide dismutase (SOD) and catalase (CAT) enzyme activities*

212 An increase in antioxidants can induce tolerance against oxidative stress. Superoxide
213 dismutase and CAT enzyme activities were improved in plants that received most of signaling
214 molecules treatment. In detail, SOD activity was increased by 45% in the H_2S+NO-supplemented
215 inflorescences (Fig 7A). Catalase activities were enhanced by 65%, 68%, and 76% against H_2S,
216 NO and H_2S+NO applications, respectively (Fig 7B).

217

218 *3.7. Proline concentration*

219 In rapidly senescent plant cells, proline increases significantly. In the present study, proline
220 levels were reduced by up to 47% in the H_2S+NO-treated inflorescences (Fig 7C).

221

222 *3.8. Correlation analysis*

223 Strong correlations were found between preharvest physiological responses and growth
224 performance (Table 1). SPAD values were significantly correlated with SS, TSP, corm mass (CM),
225 corm diameter (CD), number of leaves (NL), leaf area (LEA), days to harvest (DtH), inflorescence
226 length (IL) and number of florets (NFlo). SPAD values were significantly increased by H_2S and
227 NO treatments indicating an elicitor response that induced chlorophyll production, photosynthesis,
228 growth and flowering. Higher SS and TSP accumulated by the primary metabolism SS were
229 correlated with higher CM, CD IL and NFlo (Table 1).
230 Strong correlations were found between primary metabolism products (e.g. SS and TSP) and
231 secondary metabolites leading to anti-oxidation responses (e.g. SOD, CAT and proline) (Table 2).
232 Also, SS and TSP were negatively correlated with MDA and H_2O_2 suggesting that primary
233 metabolites helped in reduction of lipid peroxidation and overall oxidative stress (Table 2). SOD
234 and CAT productions were negatively correlated with oxidative stress indicators of MDA and
235 H_2O_2 (Table 2).
236
237 **4. Discussion**
238 The demand for cut flowers is on steady rise in the international cut flower markets. Flower
239 growers and sellers are continuously searching for new strategies to improve quality, production
240 volumes, VL, and corm yield. Senescence is a fundamental phenomenon of the developmental
241 progress ongoing on the cell tissue (Woo et al., 2018). Recent studies have demonstrated that
242 postharvest treatment with signaling molecules were able to ameliorate the oxidative stress and
243 improve VL of cut flowers. In the current experiments, gladiolus was treated with H_2S and NO to
244 enhance preharvest physiological traits that would increase the VL and improve overall quality.
245 Exogenous H_2S and NO application improved growth and corm production of gladiolus under

246 normal growing conditions. Growth improvements, as a result of H_2S and NO treatments, could
247 be related to significant increases in chlorophyll and leaf protein concentration, to accumulation
248 of soluble sugars and to significant enhancement of antioxidant potential. In this study, foliar
249 applications of H_2S and NO increased SPAD values and leaf gas exchange. These increases could
250 be associated with the increases in increased corm yield and VL performance. The results relates
251 with those presented by Ozfidan-Konakci et al. (2022) in which leaf gas exchange in Arabidopsis
252 was improved by H_2S application. Batista et al. (2018) reported the improvement of leaf gas
253 exchange and chlorophyll concentration under water deficit in response to NO application. Such
254 improvements were associated with higher protein biosynthesis that positively influenced growth,
255 flower development and corm production. Increases in chlorophyll content under H_2S and NO
256 application was found to be a physiological marker justifying the stimulation of the primary
257 metabolism.

258 Yamada et al. (2003) noted that, sugars play a vital role in conquering the incidence of
259 programmed cell death in gladiolus. Senescence is a type of developmental programmed cell death
260 (van Doorn and Woltering, 2008). H_2S and NO application regulated cut gladiolus floret
261 senescence by sugar and protein accumulation and by up-regulation of antioxidant enzymes that
262 reduced lipid peroxidation and H_2O_2. Wei et al. (2021) noted that application of H_2S to cut
263 chrysanthemum and rose plants extended VL via improving sugar and protein content, water
264 relations and antioxidant activities. Similarly, Mittal et al. (2021) reported that application of NO
265 to gladiolus spikes after harvest improved VL via improving antioxidants, proteins and sugars.
266 H_2S, NO and H_2S+NO treatments reduced ROS, or ROS markers such as lipid peroxidation and
267 helped the maintenance of the membrane stability (Bailly et al., 1996). Present results correlate
268 with Li et al. (2021), Haq et al. (2021) and Hajihashemi and Jahantigh (2022), where they indicated

that H_2S or NO had major roles in lowering lipid peroxidation and H_2O_2 in cut inflorescences of *Lilium hybrids*, *Consolida ajacis and Narcissus tazzeta,* respectively.

Soluble sugars and proteins play vital roles in osmoregulation during plant growth and development. Osmoregulators aid plant growth under stressful environments (Ozturk et al., 2021). The water-retentive ability of cut flowers is purely linked with the level of SS and TSP. In the current study, application of H_2S and NO significantly increased SS and TSP compared to the controls providing clear evidence that they helped in maintaining osmotic balance. These results are in accordance with other studies. For instance, Wei et al. (2021) reported extension of VL of cut chrysanthemums and roses as a result of SS and TSP increase after H_2S treatment. Mittal et al. (2021) noted significant improvements in VL of gladiolus inflorescences treated with NO in relation to SS and TSP increases.

The antioxidant enzyme-based defense systems are part of the vital strategy of plants against senescence and cellular damage in response to oxidative stress (Zulfiqar and Ashraf, 2022). In plant cells, protection by oxidative stress occurs via the adaptive responses of the catalytic enzymes (Zhou et al. 2014). In the current experiments, SOD and CAT activities were increased in response to foliar H_2S and NO treatments. CAT activity enhancement was recorded after postharvest NO and H_2S application to cut gladiolus, roses and chrysanthemums (Dwivedi et al., 2016; Wei et al., 2021). SOD was found to increase in response to NO and H_2S treatments and may scavenge excessive ROS during senescence of cut flowers (Dwivedi et al., 2016; Wei et al., 2021). Elimination of excessive ROS by H_2S-mediated antioxidant enzyme activities was enhanced at storage in cut daylily inflorescences (Liu et al., 2017). Although, these results provide evidence that H_2S and NO mediate antioxidant boost, studies related to preharvest treatment of H_2S or NO were not available in the literature. In the current experiments preharvest H_2S and NO

treatments induced the postharvest performance and the antioxidant response of the gladiolus inflorescences. Hence, H_2S and NO can be a useful strategy for mitigation of ROS damage during senescence of cut gladiolus inflorescences by stimulating antioxidant enzyme activities. Proline is considered a non-enzymatic antioxidant molecule having a crucial role in osmotic regulation in plants (Zulfiqar et al. 2020). Lowered proline levels in the H_2S or NO-treated cut gladiolus provided additional evidence that H_2S and NO decreased water deficit stress. Nitric oxide treatments lowered proline levels in cut gerbera (*Gerbera jamesonii*) (Shabanian et al., 2018). It was noted that under heavy metal stress conditions, H_2S and proline were coordinated to increase stress tolerance significantly (Tian et al., 2016). Both H_2S and NO have effects through thiol modifications (S-nitrosylation and S-persulfidation), and in some manner may compete for the thiol groups of proteins. Certainly, both H_2S and NO are likely to be generated spatially and temporally together, and they therefore accumulate under stress conditions in plants such as in cut flowers. Therefore, they may act at the same time and have common targets (Hancock and Whiteman, 2014; 2016). The research evidences implies that S-nitrosylation is important in NO-mediated biological activity such as improvement in vase life of cut flowers (Fig 10) (Fernando et al., 2019). Hydrogen sulfide (H_2S)-dependent protein persulfidation is also responsible in plants regarding biological activities such as postharvest improvement (Corpas et al., 2021). Common targets of both these signalling molecules are also reported in the plant cell functions (Palma et al., 2020). Collectively, preharvest treatments with H_2S and NO can be a convenient preservation strategy to maintained water status in gladiolus cut inflorescences and extend VL.

Conclusions

Combined applications of H_2S+NO greatly improved growth, physiology, and biochemical aspects in leaves and prolonged the postharvest life of gladiolus spikes. Exogenous application of H_2S and NO improved defense enzyme activities and ameliorated the oxidative stress-encouraged senescence phenomenon. The application of H_2S and NO improved corm mass and diameter by aiding the enhancement of photosynthesis. Future studies should evaluate the higher rates to see if these signaling molecules can provide even better results in postharvest performance of cut gladiolus. Moreover, H_2S and NO can react together to make a nitrosothiol and this can then act in a signalling role in its own right. Therefore, the roles of H_2S and NO are quite complex, and worth more exploration, such as done in this study.

Declaration of interest: The authors declare no conflict of interest.

Contribution: FZ conceived the idea, performed the experiment and wrote first draft. All authors improved and revised the manuscript. All authors approved the submission of final manuscript.

Acknowledgments

The authors extend their appreciation to the researchers supporting project number (RSP2023R298), King Saud University, Riyadh, Saudi Arabia.

Funding

This research was funded by researchers supporting project number (RSP2023R298), King Saud University, Riyadh, Saudi Arabia.

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Figure Legends

Figure 1. Number of leaves (A), average leaf area (mm^2; A), days to harvest (B), inflorescence length (cm; C) and number of florets (C) of gladiolus plants treated with distilled water (control), hydrogen sulfide (H_2S), nitric oxide (NO), and combination of H_2S and NO (H_2S+NO). Data shown are means ± SE (n = 15) from each treatment. Different letters above the bars represent significant differences according to Duncan's test at $p \leq 0.05$

Figure 2. Corm mass (gr; A), and corm diameter (cm; A) of gladiolus treated with distilled water (control), hydrogen sulfide (H_2S), nitric oxide (NO), and combination of H_2S and NO (H_2S+NO). Data shown are means ± SE (n = 15) from each treatment. Different letters above the bars represent significant differences according to Duncan's test at $p \leq 0.05$

Figure 3. Net CO_2 assimilation (µmol m^{-2} s^{-1}; A), stomatal conductance (mmol m^{-2} s^{-1}; B), transpiration (mmol m^{-2} s^{-1}; C) and chlorophyll (SPAD; D) of gladiolus treated with distilled water (control), hydrogen sulfide (H_2S), nitric oxide (NO), and combination of H_2S and NO (H_2S+NO). Data shown are means ± SE (n = 15) from each treatment. Different letters above the bars represent significant differences according to Duncan's test at $p \leq 0.05$

Figure 4. Vase life (days) of cut spikes of gladiolus from plants treated with distilled water (control), hydrogen sulfide (H_2S), nitric oxide (NO), and combination of H_2S and NO (H_2S+NO). Data shown are means ± SE (n = 15) from each treatment. Different letters above the bars represent significant differences according to Duncan's test at $p \leq 0.05$

Figure 5. Soluble sugars (g kg^{-1}; A), and total soluble proteins (g kg^{-1}; B) of gladiolus treated with distilled water (control), hydrogen sulfide (H_2S), nitric oxide (NO), and combination of H_2S and NO (H_2S+NO). Data shown are means ± SE (n = 15) from each treatment. Different letters above the bars represent significant differences according to Duncan's test at $p \leq 0.05$

Figure 6. Malondialdehyde (MDA) (mmol kg^{-1}; A) and hydrogen peroxide (H_2O_2) (mmol kg^{-1}; B) of gladiolus treated with distilled water (control), hydrogen sulfide (H_2S), nitric oxide (NO), and combination of H_2S and NO (H_2S+NO). Data shown are means ± SE (n = 15) from each treatment. Different letters above the bars represent significant differences according to Duncan's test at $p \leq 0.05$

Figure 7. Superoxide dismutase (SOD) (unit mg^{-1} protein; A), catalase (CAT) activities (unit mg^{-1} protein; B) and proline (µmol g^{-1} FW; C) in gladiolus treated with distilled water (control), hydrogen sulfide (H_2S), nitric oxide (NO), and combination of H_2S and NO (H_2S+NO). Data shown are means ± SE (n = 15) from each treatment. Different letters above the bars represent significant differences according to Duncan's test at $p \leq 0.05$

Figure 8. Principal component analysis (PCA) of (A) active individuals and (B) different active variables (evaluated indexes) of gladiolus supplemented with distilled water as control (CK), H_2S, NO, and H_2S+NO. (A) The score plot indicates the distribution of treatments means of 15 replications from each treatment.

Figure 9. Pearsons correlation analysis between different studies indexes. Blue and brownish colors indicate positive and negative correlations, respectively.

Figure 10. A schematic demonstration of the mechanism for H_2S and NO joint reaction to make a nitrosothiol and this can then act in a signaling role in its own right.

535 Table 1: Pearson correlations (2-tailed) between As, SPAD, soluble sugars (SS) total soluble proteins (TSP), corm mass (CM), corm
536 diameter (CD), number of leaves (NL), leaf area (LEA), days to harvest (DtH), inflorescence length (IL) and number of florets (NFlo)
537 per inflorescence. Correlation analysis was performed in SPSS v. 21.

		As	**SPAD**	**SS**	**TSP**	**CM**	**CD**	**NL**	**LEA**	**DtH**	**IL**	**NFlo**
As	*Pearson correlation*	1	0.321*	0.125	0.217	0.394*	0.069	0.333*	-0.080	-0.117	0.287	0.041
	Sig. (2-tailed)	-	0.044	0.441	0.179	0.012	0.674	0.035	0.622	0.471	0.073	0.801
SPAD	*Pearson correlation*	0.321*	1	0.479**	0.529**	0.656**	0.322*	0.319*	0.396*	-0.380*	0.438**	0.250
	Sig. (2-tailed)	0.044	-	0.002	0.000	0.000	0.042	0.045	0.012	0.015	0.005	0.125
SS	*Pearson correlation*	0.125	0.479**	1	0.666**	0.419**	0.608**	0.198	0.193	-0.369*	0.524**	0.575**
	Sig. (2-tailed)	0.441	0.002	-	0.000	0.007	0.000	0.220	0.232	0.019	0.001	0.000
TSP	*Pearson correlation*	0.217	0.529**	0.666**	1	0.387*	0.509**	0.193	0.183	-0.249	0.522**	0.322*
	Sig. (2-tailed)	0.179	0.000	0.000	-	0.014	0.001	0.233	0.259	0.141	0.001	0.043
CM	*Pearson correlation*	0.394*	0.656**	0.419**	0.387*	1	0.362*	0.056	0.270	-.0401*	0.411**	0.208
	Sig. (2-tailed)	0.012	0.000	0.007	0.014	-	0.022	0.733	0.092	0.010	0.008	0.199
CD	*Pearson correlation*	0.069	0.322*	0.608**	0.509**	0.362*	1	0.076	-0.010	-0.269	0.378*	0.326*
	Sig. (2-tailed)	0.674	0.042	0.000	0.001	0.022	-	0.764	0.950	0.093	0.016	0.040
NL	*Pearson correlation*	0.333*	0.319*	0.198	0.193	0.056	0.076	1	0.006	-0.091	0.427**	0.123
	Sig. (2-tailed)	0.035	0.045	0.220	0.233	0.733	0.764	-	0.973	0.578	0.006	0.449
LEA	*Pearson correlation*	-0.080	0.396*	0.193	0.183	0.270	-0.010	0.006	1	0.235	0.253	0.223
	Sig. (2-tailed)	0.622	0.012	0.232	0.259	0.092	0.950	0.973	-	0.145	0.116	0.167
DtH	*Pearson correlation*	-0.117	-0.380*	-0.369*	-0.249	-.0401*	-0.269	-0.091	0.235	1	-0.247	-0.107

	Sig. (2-tailed)	0.471	0.015	0.019	0.141	0.010	0.093	0.578	0.145	-	0.124	0.512
IL	*Pearson correlation*	0.287	0.438**	0.524**	0.522**	0.411**	0.378*	0.427**	0.253	-0.247	1	0.338*
	Sig. (2-tailed)	0.073	0.005	0.001	0.001	0.008	0.016	0.006	0.116	0.124	-	0.033
NFlo	*Pearson correlation*	0.041	0.250	0.575**	0.322*	0.208	0.326*	0.123	0.223	-0.107	0.338*	1
	Sig. (2-tailed)	0.801	0.125	0.000	0.043	0.199	0.040	0.449	0.167	0.512	0.033	-

538 *Correlation is significant at the 0.05 level

539 **Correlation is significant at the 0.01 level

540

541 Table 2: Pearson correlations (2-tailed) between soluble sugars (SS), total soluble proteins (TSP), MDA, H_2O_2, SOD, CAT and
542 proline. Correlation analysis was performed in SPSS v. 21.

		SS	**TSP**	**MDA**	**H_2O_2**	**SOD**	**CAT**	**Proline**
SS	*Pearson correlation*	1	0.666**	-0.628**	-0.133	0.441**	0.633**	0.498**
	Sig. (2-tailed)	-	0.000	0.000	0.413	0.004	0.000	0.001
TSP	*Pearson correlation*	0.666**	1	-0.660**	-0.196	0.427**	0.557**	-0.360*
	Sig. (2-tailed)	0.000	-	0.000	0.225	0.004	0.000	0.022
MDA	*Pearson correlation*	-0.628**	-0.660**	1	0.259	-0.390*	-0.737**	0.498**
	Sig. (2-tailed)	0.000	0.000	-	0.106	0.013	0.000	0.001
H_2O_2	*Pearson correlation*	-0.133	-0.196	0.259	1	-0.148	0.251	0.204
	Sig. (2-tailed)	0.413	0.225	0.106	-	0.362	0.118	0.207
SOD	*Pearson correlation*	0.441**	0.427**	-0.390*	-0.148	1	0.251	-0.321*
	Sig. (2-tailed)	0.004	0.004	0.013	0.362	-	0.118	0.045
CAT	*Pearson correlation*	0.633**	0.557**	-0.737**	0.251	0.251	1	-0.321*
	Sig. (2-tailed)	0.000	0.000	0.000	0.118	0.118	-	0.042
Proline	*Pearson correlation*	0.498**	-0.360*	0.498**	0.204	-0.321*	-0.321*	1
	Sig. (2-tailed)	0.001	0.022	0.001	0.207	0.045	0.042	-

543 *Correlation is significant at the 0.05 level

544 **Correlation is significant at the 0.01 level