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Hydrogen sulphide increase the tolerance to alkalinity stress in cabbage plants (*Brassica oleracea* L. 'Bronco')

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ABSTRACT

The present study investigates how the application of hydrogen sulphide (0.5 mM of NaHS) in *Brassica oleracea* L. 'Bronco' influences the processes involved in glutathione homeostasis and tolerance to alkaline stress (50 mM $NaHCO_3$:Na_2CO_3). According to our results, alkaline stress increases the $O_2\cdot^-$ content, lipid peroxidation, and the activities of the enzymes glyoxalase I (Gly I) and glyoxalase II (Gly II) that detoxify methylglyoxal (MG) while decreasing biomass, the activity of superoxide dismutase (SOD), the activity of enzymes involved in glutathione (GSH) synthesis and in the AsA-GSH cycle, as well as the content in reduced glutathione and the different forms of ascorbate (AsA). On the other hand, the application of NaHS improved the antioxidant response, inducing SOD activity and improving processes involved in glutathione homeostasis, boosting the reduced GSH content as well as the activity of key enzymes in glutathione synthesis and in the AsA-GSH cycle. Consequently, the application of H_2S in the form of NaHS at a concentration of 0.5 mM could fortify the tolerance of *Brassica oleracea* L. 'Bronco' against alkaline stress.

1. Introduction

Alkaline stress can be defined as a stress by alkaline salts, such as $NaHCO_3$ and Na_2CO_3. Therefore, it is important to specify that this type of stress is caused by alkaline salts and not by neutral salts (NaCl), which causes what is commonly known as "salinity stress" (Shi and Sheng, 2005; Shi and Wang, 2005). However, the effects of the two types of stress can be similar in some cases, but several studies have shown that alkaline stress provokes more severe damage than does salinity stress (Kawanabe and Zhu, 1991; Wang et al., 2008).

Alkaline stress, very common in arid and semiarid areas, lowers agricultural productivity in these regions, where agriculture is normally intensive. Worldwide, 10% of cultivated soils are subject to alkaline stress (Tanji, 2002), and the FAO predicts that by the year 2050, the reduction in land available for agriculture for the presence of these types of stress will exceed 50% (Jin et al., 2008).

The high pH and the massive accumulation of Na^+ provoked by alkaline stress can lead to a lack of protons and to the destruction or inhibition of the electrochemical gradients/potentials in root cells, altering many physiological functions, such as water and ion uptake (Wang et al., 2011a; Wang et al., 2011b). Due to these alterations in physiological functions of the root, alkaline stress (pH > 8.5) inhibits growth and photosynthesis. Inhibited photosynthesis results from fewer photosynthetic pigments, often due to under alkaline stress to a lower uptake of Mg^{2+} caused by its precipitation in the rhyzosphere (Li et al., 2010). Other authors contend that P limitation due to its precipitation under alkaline conditions may diminish CO_2 intake for photosynthesis (Fredeen et al., 1990). The reduced CO_2 intake due to this type of stress causes oxidative damage in cells and alters antioxidant metabolism, generating reactive oxygen species (ROS). Excess electrons produced during photosynthesis are transferred to O_2 molecules and these produce ROS (Cakmak, 2005).

The ROS released adversely affect biological structures, including DNA damage, amino acid oxidation, and lipid peroxidation (Asada, 1999; Johnson et al., 2003). ROS react with cell membranes, especially thylacoid membranes, which are rich in unsaturated fatty acids. Lipid peroxidation breaks up these fatty acids, disrupting their function in the membrane, disturbing fluidity, breaking lipid bonds, and inactivating membrane enzymes (Miyake et al., 2005).

To avoid this type of damage, plants have ROS-detoxification mechanisms which can be divided into: enzymatic systems, composed of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR); and non-enzymatic systems, made up of antioxidant compounds such as phenolic compounds (e.g. phenols, flavonoids, carotenoids), ascorbic acid (AsA), and glutathione (GSH) (Shalata et al., 2001; Reddy et al., 2004).

Therefore, treatments to stimulate the antioxidant response in

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plants could be effective to reduce the damage provoked by alkaline stress. Recently, research has focused on the use of fortification programmes with ions such as iodine, selenium, and silica to reduce or alleviate abiotic stress. In addition to the ions mentioned above, these treatments also use exogenous H_2S.

For many years, H_2S has been considered a phytotoxin, since high doses of H_2S applied to greenhouse crops cause leaf injury, defoliation, growth reduction, and death in sensitive species such as alfalfa (*Medicago sativa* L.) and lettuce (*Lactuca sativa*), among others (Thompson and Kats, 1978). However, recent studies indicate that H_2S application at certain doses can act as a signalling molecule, bolstering tolerance to different types of abiotic stress by inducing the antioxidant response, especially stimulating GSH synthesis. These results refer to heat stress in *Zea Mays*, drought stress in *Triticum aestivum* L., cold stress in *Musa* spp., stress from excess arsenates in *Pisum sativum* L., stress from heavy metals with lead in *Brassica napus*, and salt stress in strawberry (Fragaria x ananassa cv. Camarosa) (Shan et al., 2011; Christou et al., 2013; Li et al., 2014; Ali et al., 2014; Luo et al., 2015; Singh et al., 2015).

In fact, greater tolerance against these types of stress from the induction of the antioxidant response can be achieved by altering GSH homeostasis, since this compound is fundamental in this process. GSH, a tripeptide with a thiol group composed of three amino acids (L-cysteine, acid L-glutamic, and glycine), is consumed in numerous redox reactions to combat oxidative stress, giving rise to its oxidized form GSSG (Mishra et al., 2008). The enzyme serine acetyltransferase (SAT) is considered the key enzyme in cysteine synthesis, providing one of the substrates of GSH synthesis (Droux, 2004). GSH is synthesised in two steps that depend on ATP catalysed by the enzymes gluatmylcysteine synthetase (γ-ECS; the enzyme that limits the speed of the process) and the enzyme glutathione synthetase (GS) (Noctor and Foyer, 1998; Tripathi et al., 2012). First, γ-ECS catalyses the formation of the peptide bond between the γ-carboxyl group of glutamate and the α-amino cysteine, and then GS catalyses the formation of the peptide bond between the cisteinyl group of γ-glutamylcysteine and the α-amine group of glycine (Flocco et al., 2004). It has been demonstrated that the overexpression of the enzyme serine acetyltransferase SAT increased resistance to oxidative stress in transgenic tobacco plants (Blaszczyk et al., 1999). On the other hand, recent studies show that H_2S can act in the regulation of GSH synthesis and accumulation, increasing γ-ECS activity under conditions of water stress in wheat plants (Shan et al., 2011).

The GSH has diverse functions in the protection against oxidative stress, acting in the ascorbate-glutathione cycle, which is fundamental in antioxidant defence. In this cycle, APX reduces hydrogen peroxide (H_2O_2) to water using AsA as the electron donor, in such a way that this latter compound is oxidized, converting to dehydroascorbate (DHA), which in turn is again reduced to AsA using reduced GSH as the electron donor. On the other hand, the GSSG formed is transformed again into reduced GSH in the reaction catalysed by the NAD(P)H-dependent enzyme GR (Gomes et al., 2013). Some researchers hold that the ascorbate-glutathione cycle plays a prominent role in tolerance against alkaline stress (Liu et al., 2015). On the other hand, there are studies stating that H_2S pretreatments increase enzyme activities in ROS detoxification, such as those involved in the ascorbate-glutathione cycle: APX, GR, DHAR, and one of the key enzymes in GSH synthesis, such as γ-ECS under salinity-stress conditions (Shan et al., 2014). Under these conditions, it has also been observed that the decrease in the AsA/DHA and GSH/GSSG quotients was mitigated after H_2S application (Shan et al., 2014).

In addition, GSH actively participates in the detoxification of MG, which can cause oxidative stress that closely resembles that caused when the ROS concentration rises in plant cells (Wang et al., 2009; Desai et al., 2010). MG is a cytotoxic compound generated through the glucolysis pathway in eukaryote cells (Yadav et al., 2005a), and its overproduction can be generated through fatty acids or the metabolism of amino acetone (Casazza et al., 1984). The small quantity of MG produced under normal growth conditions can easily and quickly be metabolised by the glyoxalase system of the plant. However, different stress conditions can trigger greater production and thus accumulation of MG. Depending on the species, the MG concentration can rise 2- to 6-fold in response to the different types of stress, such as salinity, drought, or cold (Yadav et al., 2005a,b).

Many works show that the MG content increases under osmotic stress and that GSH metabolism is important in the detoxification of this compound under these conditions (Veena et al., 1999). MG is detoxified primarily by maintaining the homeostasis of GSH through the glyoxalase system. This involves the activity of two enzymes: glyoxalase I (Gly I) and glyoxalase II (Gly II). Gly I uses a molecule of reduced GSH to convert MG into S-d-lactoylglutathione (SLG). Afterwards Gly II converts SLG into d-lactate and a molecule of reduced GSH that is recycled to enter the system again (Yadav et al., 2008). Transgenic tomato (*Solanum lycopersicum* L.) plants that overexpress Gly I show significant tolerance to MG and to high NaCl concentrations. In these plants, Gly I expression reportedly increases in response to salt stress, osmotic stress, and phytohormone stimuli (Espartero et al., 1995). Also, it has been shown that the application of NaHS (0.1 mM) can spur the activity of Gly I and Gly II when an excess of MG is generated under conditions of Cd stress in rice plants (*Oryza sativa* L. cv. BRRI dhan52).

Finally, another enzyme that also determines the homeostasis of GSH is glutathione peroxidase (GPX), which uses the GSH pool as a substrate to detoxify H_20_2 produced under stress conditions (Anjum et al., 2012). For example, the overexpression of the enzyme GPX in transgenic tobacco plants increased seedling growth under different stress conditions of cold (10 °C), heat (30 °C), and salinity (100 mM of NaCl) (Roxas et al., 2000). Li et al. (2014) have reported increased GPX activity after pretreatment of *Zea mays* plants with NaHS (3 mM) under heat-stress conditions.

In view of the above, in the present work, we seek to determine the alterations caused in antioxidant metabolism, and specifically GSH homeostasis under severe alkaline stress. In this sense, we determine whether NaHS application could fortify the antioxidant capacity and promote GSH homeostasis in *Brassica oleracea* L. 'Bronco' plants under alkalinity stress, with the final aim of considering the use of this compound as a fortifier in areas where the levels of alkaline salts in the soil limit yield and quality in these crops.

2. Material and methods

2.1. Crop management and experimental design

Seeds of *B. oleracea* cv. Bronco (genotype sensitive to alkalinity) were germinated and grown for 30 days in cell flats of 3 cm × 3 cm × 10 cm filled with a perlite mixture substratum. The flats were placed on benches in an experimental greenhouse located in Southern Spain (Saliplant S.L., Motril, Granada). After 30 days, the seedlings were transferred to a growth chamber under the following controlled environmental conditions: Relative humidity 50%; Day/night temperatures 25/18 °C; 16/8 h photoperiod at a photosynthetic photon flux density (PPFD) of 350 μmol m^{-2} s^{-1} (measured at the top of the seedlings with a 190 SB quantum sensor, LI-COR Inc., Lincoln, Nebraska, USA). The plants were acclimating to these conditions during 8 days. Under these conditions the plants were grown in hydroponic culture in lightweight polypropylene trays (60 cm diameter top, bottom diameter 60 cm and 7 cm in height) of 3 L volume. Throughout the experiment the plants were treated with a growth solution made up of 4 mM KNO_3, 3 mM $Ca(NO_3)_2{\cdot}4H_2O$,2 mM $MgSO_4{\cdot}7H_2O$, 6 mM KH_2PO_4, 1 mM $NaH_2PO_4{\cdot}2H_2O$, 2 μM $MnCl_2{\cdot}4H_2O$, 10 μM $ZnSO_4{\cdot}7H_2O$, 0.25 μM $CuSO_4{\cdot}5H_2O$, 0.1 μM $Na_2MoO_4{\cdot}2H_2O$, 5 mg L^{-1} Fe-chelate (Sequestrene; 138 FeG100) and10 μM H_3BO_3. This solution, with a pH of 5.5–6.0, was changed every three days.

The treatments were initiated 38 days after germination and were