**Application of exogenous salicylic acid induces resistance in pakchoi** **(*Brassica campestris* ssp. *chinensis* Makino) against *Plasmodiophora brassicae***

**Running title: Salicylic acid induces clubroot in pakchoi**

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**Abstract**

Salicylic acid (SA) is a plant hormone that plays critical roles in defense against diseases. To investigate the mechanism of SA-induced resistance against clubroot, we analyzed the effect of SA on growth of clubroot-sensitive plants, alteration of antioxidative system, and related gene expression. A clubroot-sensitive pakchoi cultivar, xinxiaqing, was treated with *P. brassicae* and/or 0.6 mM exogenous SA. Results revealed that clubroot significantly affected pakchoi growth and enhanced reactive oxygen species (ROS) contents and membrane lipid peroxidation. After 0.6 mM exogenous SA treatment, both clubroot incidence rate and disease index were decreased in inoculated plants. Exogenous SA also increased the activities of superoxide dismutase (SOD), ascorbic acid-peroxidase (APX), catalase (CAT), and glutathione reductase (GR). Additionally, the production rate of malondialdehyde (MDA), hydrogen peroxide (H2O2), and superoxide anion (O2· –) was inhibited by SA. The expression levels of genes, encoding antioxidant enzymes ,were decreased in group Inoculation+SA plants. Collectively, we conclude that 0.6 mM SA contributes to induce plant resistance to clubroot by increasing activities of antioxidant enzymes, abilities of osmotic regulation, and ROS scavenging to decrease clubroot-induced damage in pakchoi.

**Key words: exogenous SA, pakchoi, clubroot, resistance induction, antioxidant system**

**Introduction**

Clubroot is a worldwide soil-born disease that is caused by *Plasmodiophora brassicae* Woron (*P. brassicae*) infection (Howard et al., 2010). *P. brassicae* can infect over 100 Brassica species, including *Brassica napus* L., *Brassica rapa pekinesis*, *Brassica oleracea* L., and *Brassica rapa* L., resulting in great economic loss. The life cycle of *P. brassicae* is mainly composed of primary phase taking place in root hairs and secondary phase taking place in cortical and stele cells (Ingram and Tommerup, 1972; Lemarié et al., 2015). In recent years, damage of clubroot becomes more and more serious. According to the statistics, clubroot incident area had already up to 4666.7 hm2 only in Shanghai (China) in 2017 (supplied by Shanghai Agricultural Technology Extension Service Center). To successfully control clubroot, many researches pay much attention to control clubroot, such as chemical control, crop rotation, and genetic control (Howard et al., 2010).

To overcome pathogen infections, plants have developed a defense system comprising of pathogen-associated molecular patterns (PAMP)-triggered immunity (PTI) and resistance (R) proteins associated pathogen effector-triggered immunity (ETI) (Jones and Dangl, 2006; An and Mou, 2011). Following activated PTI or ETI, the mobile signals, produced in local infected tissues, moves to distal tissues, activating systemic acquired resistance (SAR) (An and Mou, 2011). SA is a small phenol in plants and well-studied as a signal molecular during plant immunity response, although it also plays essential roles in in cellular signal transduction, plant growth and development, and abiotic stress (Morris et al., 2000; Vlot et al., 2009; Spormann et al., 2019)

Oxidative stress acts as a result of various stresses, including biotic stress. The primary reason for oxidative stress is excessive accumulation of reactive oxygen species (ROS), produced during aerobic metabolism and damage plant cells (Sunkar et al., 2003; Apel and Hirt, 2004). To avoid ROS damage, plants evolved two systems comprising of non -enzymatic and enzymatic ROS scavenging mechanisms (Apel and Hirt, 2004). The involved enzymes mainly include superoxide dismutase (SOD), ascorbic acid-peroxidase (APX), glutathione peroxidase (GPX), catalase (CAT), and glutathione reductase (GR) (Apel and Hirt, 2004). Overproduction of ROS, beyond plant scavenging ability, further results in plant hypersensitive reaction (HR) and damages to cells. Ozone (O3) and superoxide anion (O2· – ) are reported to be accumulated in an *Arabidopsis* mutant, *radical-induced cell death 1* (*rcd1*), resulting in cell death (Overmyer et al., 2000). Contrarily, overexpression of chloroplast SOD in chloroplast enhanced plant resistance to oxidative stress (Allen, 1995).

Numerous evidences have revealed that SA is an effective strategy to inhibit clubroot disease. It has been hypothesized that SA can be converted to biologically inactive methyl salicylate by *P. brassicae* (Ludwig-Müller et al., 2015). Exogenous SA was reported to suppress clubroot in *Arabidopsis* (Agarwal et al., 2011). Similar results were also observed in broccoli (*B. oleracea)*, *Arabidopsis* Bur-0 accession(Lovelock et al., 2013; Lemarié et al., 2015). Additionally, SA related genes were differentiated expressed in *B.rapa* ssp. *Chinensis* inoculated with *P. brassicae* (Chu et al., 2014). However, the mechanism of SA repress clubroot still remains unclear.

Researches have established that exogenous SA induced resistance to abiotic and biotic stresses, possibly through regulating ROSmetabolism. Plants treated with SA increased APX and GR activity against temperature stress (Wang and Li, 2006). Application of benzothiadiazole S-methyl ester (BTH), a SA analog, induced wheat defense-related genes overexpression in wheat (Pasquer et al., 2005). Moreover, SA application induced PR-proteins in *Solanum melongene L*. against *Verticillium dahlia* Kleb (Mahesh et al., 2017). SA was also found to induce rubber tree against *Phytophthora palmivora* through increasing CAT, peroxidase (POD), and phenylalanine ammonialyase (PAL) activities (Deenamo et al., 2018). The latest study revealed that SA application enhanced two tomato cultivars resistance to *Tomato yellow leaf curl virus* (TYLCV) by increasing POD and APX activities (Li et al., 2019). A recent research showed that exogenous SA decreased clubroot symptoms in two *Arabidopsis* accessions (Lemarié et al., 2015). Conversely, degradation of endogenous SA enhanced Ralstonia solanacearum virulence in tobacco (Lowe-Power et al., 2016). Moreover all these researches suggested that SA-mediated increased clubroot resistance may depend on ROS pathway in pakchoi.

At present, there are a few of pakchoi cultivars resistance to clubroot and most cultivars exhibit susceptibility to clubroot, which bring challenges to growers and breeders. Our previous researches have revealed 0.6 mM SA have best effectivity to control clubroot (Zhu et al., 2017). However, the underlying molecular mechanism was not clear. In this study, we found that pakchoi exhibited increased clubroot resistance after exogenous SA, consistent with the previous result (Zhu et al., 2017). ROS contents, antioxidant enzyme activities, and related gene expression were altered after SA treatment. We demonstrated that exogenous SA treatment increases antioxidant enzyme activities to scavenge over-produced ROS level, resulting in increased clubroot resistance.

**Materials and Methods**

**Plant Materials**

Xinxiaqing, a pakchoi cultivar and mainly grew in Shanghai, was used for analysis and supplied by Protected Horticultural Research Institute, Shanghai Academy of Agricultural Science (SAAS). The *P. brassicae* (physiological race 7) was isolated from infected roots grew in Hongyang Farm in Qingpu District, Shanghai (Zhu et al., 2019).

***P. brassicae* and SA treatment**

*P. brassicae* infected pakchoi roots with galls were harvested from fields and stored at -20 oC until required. Diseased roots were homogenated and mixed with sterilized soil, keeping with 2×108 spores per gram soil with hemocytometer. Pakchoi seeds were sowed on soil (group Control) or mixed soil with root homogenate (group Inoculation) in growth chamber, which was set to 28±1oC day/20±1oC night and 12 h light / 12 nigh. Water content of two kind soils was 60%. Ten days after germination (DAG), seedlings (group Control and group Inoculation) were treated with water or 0.6 mM SA (group Control+SA or group Inoculation+SA, SA was diluted in ethanol/water) with one time every day for four days. Forty DAG, plants were used to calculate disease index and disease incidence rate. The third leaf was collected for RNA extraction, ROS content measurement, soluble protein content measurement, proline content measurement, and antioxidant enzyme activities.

**Disease discovery**

Disease symptoms were discovered at forty DAG. Clubroot grading was described previously (Huang et al., 2012). Grade 0：no symptoms; Grade 1: very small galls in lateral roots or primary roots, which do not affect main root growth ; Grade 3: a few of galls are found obviously in primary roots and gall size is 2-3 times than transection of basal part of stem; Grade 5: severe galls are found in lateral roots and primary roots, gall size is over 4 times than transection of basal part of stem, which affect plant growth (Huang et al., 2012).

Disease incidence rate (DIR) = (number of disease plants/total number of plants) X 100%

Disease index (DI)=∑(disease grade X number of disease plants per grade) X 100 / (total number of plants)

Induced resistance index (IRI) =｛(DI of inoculated plants- DI of inoculated plants with exogenous SA) / DI of inoculated plants｝X100%。

**Measurement of proline content and soluble protein**

Proline content was measured according to the method of Funck et al. (Funck et al., 2008). Proline was extracted with sulfosalicylic acid, followed by acidic ninhydrin reagent. The mixture was extracted with toluene. The tolune supernatant was used to detect A520. Standard curves from 0 to 10 mM proline treated in the same way was made to calculate proline content.Soluble protein content was detected using Coomassie Brilliant Blue G-250 (Bradford, 1976).

**Measurement of ROS content**

Malondialdehyde (MDA) content represents the damage of ROS to membrane lipids and was measured with thiobarbituric acid (TBA) method previously described (Jiang and Zhang, 2001). H2O2 content was measured by monitoring the absorbance of titanium–peroxide complex at 415nm described by Brennan and Frenkel (1977). O2· – content was measured as described by Jang and Jiang and Zhang (2001). In presence of O2· –, nitrite is finally synthesized from hydroxylamine. A standard curve of NO2 - was used to calculate O2· – content.

**Measurement of antioxidant enzyme activities**

SOD activity was measured with nitro blue tetrazolium (NBT) according to the method of Jiang and Zhang (Jiang and Zhang, 2001). One unit SOD activity was determined to cause 50% inhibition of the reduction of NBT at 560 nm. CAT and APX activity was measured with the method of Jiang and Zhang (Jiang and Zhang, 2001). The reaction buffer, containing 50 mM potassium phosphate buffer (pH7.0), 10 mM H2O2, and enzyme extract, was used to detect the absorbance at 240 nm for measuring CAT activity. APX enzyme extract was added to reaction buffer containing 50mM potassium phosphate buffer (pH7.0), 0.5 mM ascorbate (ASC), 0.1 mM H2O2. The decrease of APX activity in A290 for 1 min was measured in the mixed solution at 25oC. GR activity was measured with the oxidation of NADPH at 340 nm (Jiang and Zhang, 2001). The absorbance at 340 nm without NADPH was used to make corrections.

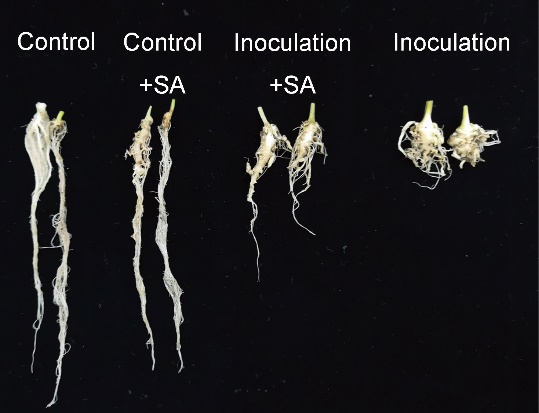
**RNA extraction and Gene expression**

To detect expression levels of *SOD*, *CAT*, *APX*, and *GR*, total RNA was extracted with RNAiso plus and tested by Nanodrop 2000 (Thermo Scientific). Then cDNA was synthesized with HiScript II Q RT SuperMix for qPCR(+gDNA wiper) (Vazyme), after which qRT-PCR was performed with QuantiFast ® SYBR® Green PCR Kit (Qiagen). Fluorescence was detected by LightCycler® 480 II (Roche). Gene expression level was calculated by 2—ΔΔCt method. *Actin* was used as reference gene. All primers used were listed in Supplementary Table 1.

**Results**

**The effects of SA on induced-clutroot-resistance and growth of pakchoi**

To explore whether SA increases pakchoi resistance to clubroot, seedlings having one leaf and one bud were treated with 0.6mM SA or water. Within Group Control, there was no galls occurred in roots (Figure 1). Plant height, maximum leaf area, and fresh weight did not changed with SA application (Table 1). Within Group Inoculation, abnormal root system occurred with large galls without SA (Figure 1). DIR and DI of Group Inoculation were up to 66.15% and 36.16% , respectively (Tabel 1). Plant height, maximum leaf area, and fresh weight on the ground were reduced by 27.40%, 39.26%, and 58.61%, respectively compared with those of Group Control (Table 1). However, after 0.6 mM SA treatment for four days, DIR and DI were only up to 25.51% and 13.45% compared to those of Group Control. DIR was reduced by 61.42% compared to that of Group Inoculation, and DI was reduced by 62.80%. Additionally, Plant height, maximum leaf area, and shoot fresh weight on the ground were only reduce by 17.22%, 19.42%, and 21.48% (Table 1). These data revealed that exogenous SA effectively released the inhibition of clubroot on pakchoi growth.



**Figure 1 Forty-day old plant roots of group Control, Control+SA, Inoculation+SA, and Inoculation**. SA concentration was 0.6 mM with four-day continuous treatment.

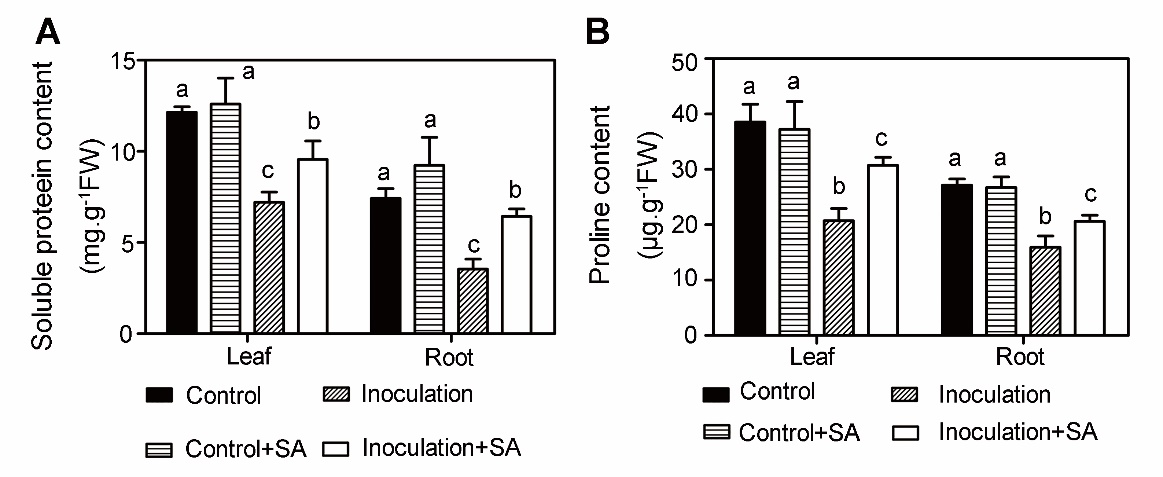
Table 1 Effect of SA on the inducted-resistance and growth of pakchoi under *P. brassicae.*

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Group | DIR(%) | DI(%) | IRI (%) | Plant height  (cm) | Maximum leaf area (cm2) | Shoot fresh weight (g) |
| Control | - | - | - | 13.65±0.51a | 25.65±0.11 a | 6.33±0.07 a |
| Inoculation | 66.15±1.07a | 36.16±2.03a | - | 9.91±0.12 c | 15.58±0.13 c | 2.62±0.02 c |
| Control+SA | - | - | - | 13.30±0.41a | 24.47±0.21a | 6.21±0.03 a |
| Inoculation+SA | 25.51±2.43b | 13.45±0.05b | 61.21±0.017 | 11.30±0.46 b | 20.67±0.20 b | 4.97±0.11 b |

Group Control or Control + SA: ten-day-old seedlings sowed in soil treated with water or 0.6mM SA, respectively; group Inoculation or Inoculation+SA: ten-day-old seedlings sowed in mixed soil (with *P. brassicae*) treated with water or 0.6 mM SA, respectively. The calculation of DIR and DI were presented in methods. DIR: disease incidence rate; DI: disease index. Data shown represent mean ± SD (n > 190 ). Different letters indicate significance (Tukey’s HSD test, P < 0.05).

**Effect of SA on proline and soluble protein contents in pakchoi**

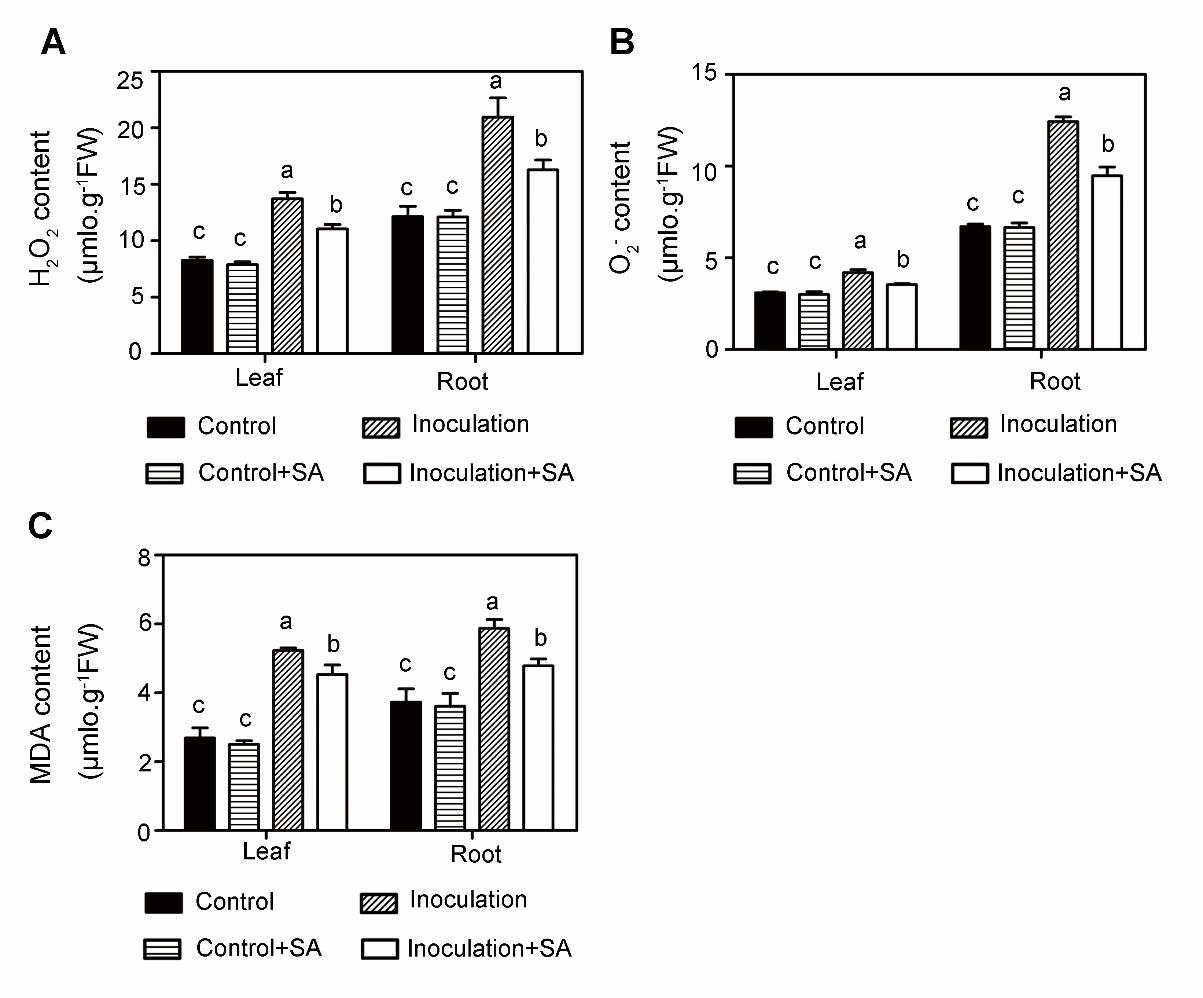
AS plant resistance to stresses can be expressed by proline and soluble protein levels, therefore, we detected the proline and soluble protein contents after SA application in *P. brassicae* – inoculated pakchoi. Soluble protein contents of group Inoculation were 7.21 mg.g-1 FW in leaves and 3.55 mg.g-1 FW in roots (Figure 2A). Proline contents were 20.72 mg.g-1 FW in leaves and 15.94 mg.g-1 FW in roots (Figure 2B). After 0.6 mM SA application, both soluble protein and proline contents were increased. Soluble protein contents were up to 9.56 mg.g-1 FW in leaves and 6.43 mg.g-1 FW in roots (Figure 2A). Proline contents were increased to 30.74 mg.g-1 FW in leaves and 20.61 mg.g-1 FW in roots (Figure 2B). These results showed that *P. brassicae* inoculation reduced soluble protein and proline contents while SA increased soluble protein and proline contents in *P. brassicae*-infected pakchoi.

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**Figure 2. Soluble protein and proline contents in pakchoi.** Soluble protein **(A)** and proline contents **(B)** in leaves and roots were presented. The third leaves were harvested for analysis. Data shown represent mean ± SD (n = 3). Different letters indicate significance (Tukey’s HSD test, P < 0.05).

**Effect of SA on ROS and MDA contents in *P. brassicae*-inoculated pakchoi**

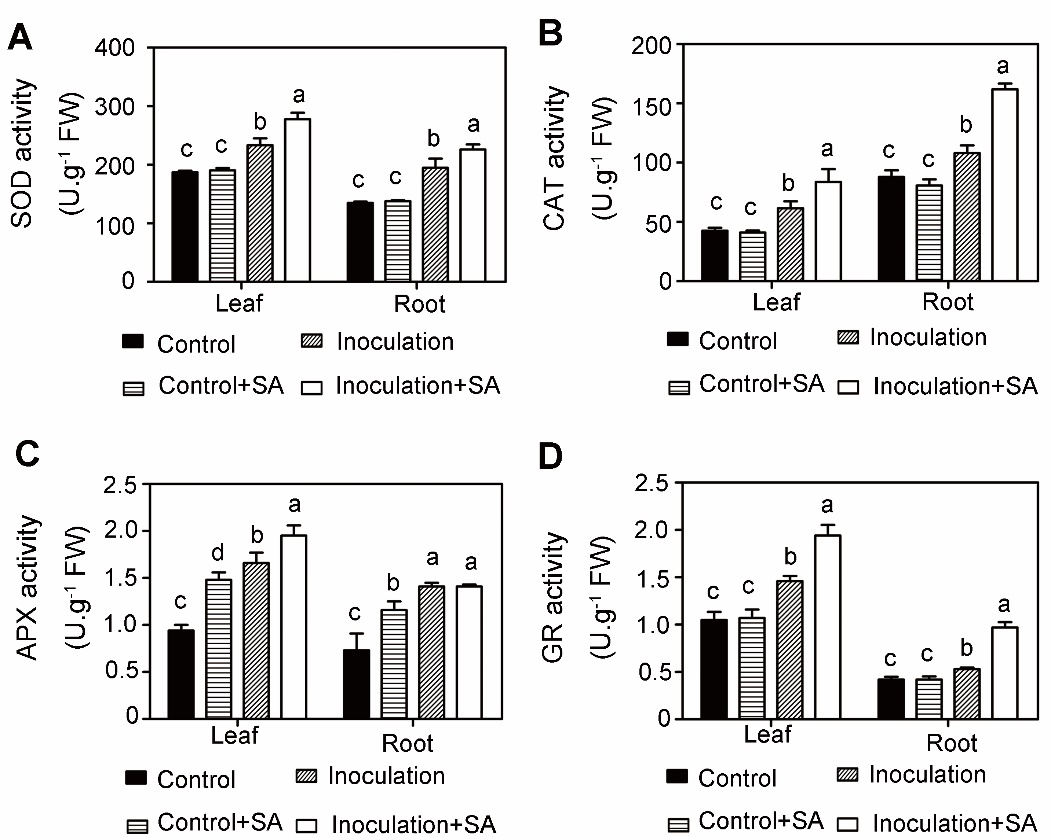
When suffer from biotic or abiotic stresses, plants will produce more ROS further leading to oxidative stress to cells. Accumulation of ROS causes cell membrane lipid peroxidation and cell death (Sunkar et al., 2003). MDA is a byproduct of membrane lipid peroxidation and its content reflects the degree of membrane lipid peroxidation (Sunkar et al., 2003). Firstly, we detected ROS content expressed by H2O2 and O2· –  contents in plants. In group Control, H2O2 contents were 8.25 μmol.g-1 FW in leaves and 12.13 μmol.g-1 FW in roots (Figure 3A). O2· – levels were 3.09 μmol.g-1 FW in leaves and 6.71 μmol.g-1 FW in roots (Figure 3B). MDA contents were 2.69 μmol.g-1 FW in leaves and 3.73 μmol.g-1 FW in roots (Figure 3C). However, ROS and MDA contents of group Inoculation were distinctly increased. H2O2, O2· –, and MDA contents were up to in 13.72 μmol.g-1 FW and 20.94 μmol.g-1 FW , 4.20 μmol.g-1 FW and 12.43 μmol.g-1 FW , and 5.23 μmol.g-1 FW and 5.87 μmol.g-1 FW, in leaves and roots, respectively (Figure 3). With SA application, H2O2,O2· – , and MDA contents of group Inoculation+SA were reduced to 11.06 μmol.g-1 FW and 16.28 μmol.g-1 FW, 3.54 μmol.g-1 FW and 9.48 μmol.g-1 FW, and 4.53μmol.g-1 FW and 4.78, in leaves and roots, respectively (Figure 3). These data revealed that *P. brassicae* inoculation significantly promoted ROS and MDA production and exogenous SA application inhibited ROS and MDA production to protect plants.



**Figure 3 SA effect on ROS and MDA contents in pakchoi.** H2O2**(A)**, O2· – **(B)**, and MDA **(C)** contents in pakchoi were measured among four groups. Forty-day old leaves or roots were harvested for analysis, respectively. Methods were detailed described in Materials and Methods. Data shown represent average ± SD. Different letters indicate significance (Tukey’s HSD test, P < 0.05).

**Effect of SA on antioxidant enzymes activities**

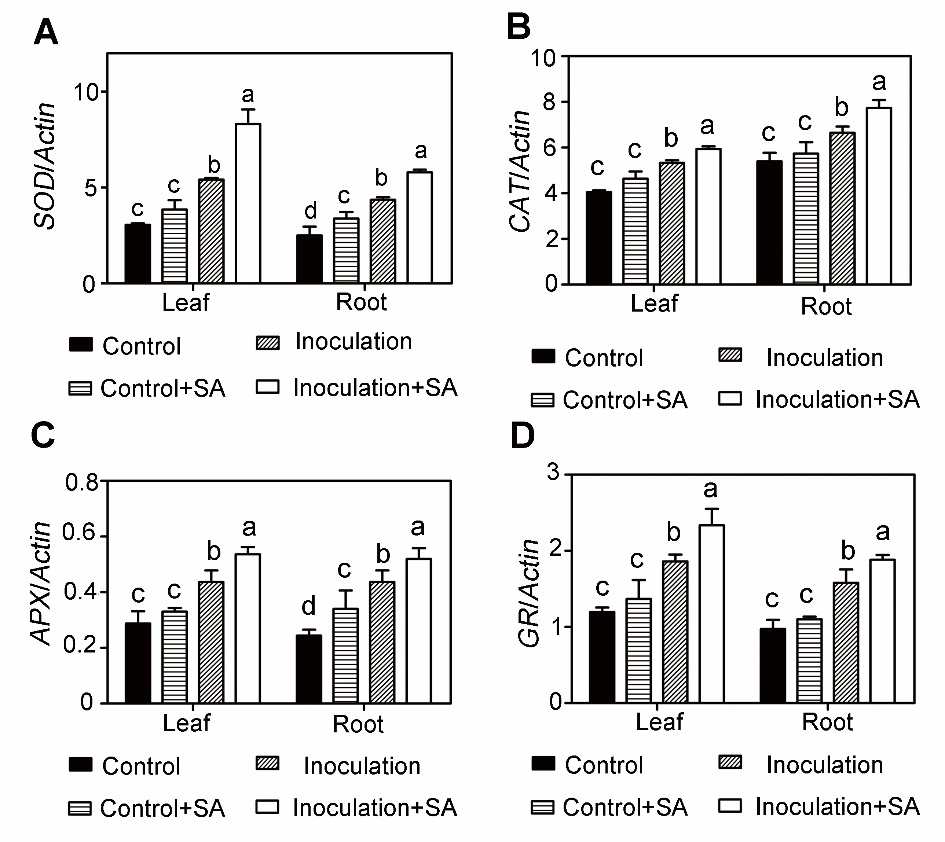
To response to stress, plants continuously adjust to their metabolism to adapt to environment, including increased activities of antioxidant enzymes to scavenge over produced ROS. Between groups without inoculation, SA treatment did not change SOD and GR activities but increased APX activities both in leaves and roots (Figure 4). In group Inoculation, SOD activities were 233.62 U.g-1 FW in leaves and 194.74 U.g-1 FW in roots, CAT activities were 61.53 U.g-1 FW in leaves and 108.13 U.g-1 FW in roots, APX were 1.66 U.g-1 FW in leaves and 1.41 U.g-1 FW in roots, and GR were 1.46 U.g-1 FW in leaves and 0.53 U.g-1 FW in roots (Figure 4). However, treated with 0.6 mM SA after inoculation, SOD activities were up to 277.82 U.g-1 FW in leaves, 226.09 U.g-1FW in roots, CAT were up to 83.80 U.g-1FW and 161.84 U.g-1FW, and GR were up to 1.94 U.g-1FW and 0.97 U.g-1FW, respectively (Figure 4). APX activity was up to 1.95 U.g-1FW in leaves but did not change in root compared to that of group Inoculation (Figure 4). Increased antioxidant enzyme activities contributes to scavenging ROS and protect plants. These data revealed that SA induced plants resistance to clubroot through antioxidant system.



**Figure 4 Antioxidant enzyme activities in pakchoi.** SOD **(A)** , CAT **(B)** , APX **(C)** , and GR **(D)** activities in pakchoi were measured among four groups at 40 DAG. Data shown represent average ± SD (n = 3). Different letters indicate significance (Tukey’s HSD test, P < 0.05).

**Effect of SA on antioxidant gene expression**

Since activities of antioxidant enzymes in pakchoi were altered after inoculation and SA application, we further detected antioxidant genes expression levels to analysis potential mechanism of SA – induced clubroot resistance in pakchoi. qRT-PCR analysis revealed that exogenous SA did not alter *SOD*,*CAT*, *APX*, and *GR* expression levels both in leaves and roots without inoculation (Figure 5). Compared to group Control, significantly increased gene expression levels were observed in group Inoculation (Figure 5). When plants of group Inoculation were treated with SA, *SOD*, *CAT*, *APX*, and *GR* expression levels were increased significantly compared to that of plants of group Inoculation (Figure 5). These data revealed that exogenous SA promoted antioxidant enzyme biosynthesis through upregulating related genes expression, resulting in increased clubroot resistance.



**Figure 5 Relative gene expression levels in roots and leaves.** SOD **(A)**, CAT **(B)**, APX **(C)**, and GR **(D)** expression levels in different groups were detected. Data shown represent average ± SD (n = 3). Different letters represent significance (one-way anova, P < 0.05).

**Discussion**

SA , as a plant hormone, has been established to promote plant growth and development, induce resistance to temperature and disease stresses. Exogenous SA has been verified to be beneficial in plant growth against abiotic and biotic stresses (Mabood and Smith, 2007). SA induces plant resistance to virus, fungi, and bacteria infection, which has been verified in tobacco and wheat (Pasquer et al., 2005; Lowe-Power et al., 2016). In this study, inoculation with *P. brassicae* impacted plant height, maximum leaf area, and fresh weight. Our previous study revealed that different concentrations of exogenous SA application have different effects on clubroot incidence in pakchoi (Zhu et al., 2017). Clubroot incidence rate of plants treated with 0.6 mM SA was lower than that of plants treated with 0.2 mM, 0.4 mM or 0.8 mM SA (Zhu et al., 2017). This suggests that 0.6 mM exogenous SA may work best in controlling clubroot. Hence, we employed 0.6 mM exogenous SA, after inoculation, leading to decreased plant DIR, increased resistance to clubroot, and promoted plant growth (Figure 1 and Table 1).

Under normal conditions, the production and scavenging of cellular ROS keep dynamic balance. When plants suffer from stresses, the balance is broken and ROS are accumulated, resulted from production rate is higher than scavenging rate by antioxidant enzymes. Our study revealed that exogenous SA inhibited the production rate of H2O2 and O2· –, as well as MDA content. ROS scavenging mainly depends on antioxidant enzymes and antioxidants. SOD, CAT, GR, and APX are key antioxidant enzymes required for ROS scavenging and preventing membrane peroxidation. These four enzymes increased their activities with SA application in plants inoculated with *P. brassicae* (Figure 4). SOD converts O2· – into H2O2 (Apel and Hirt, 2004).The decreased O2· – might result from enhanced SOD activity induced by exogenous SA (Figure 3B and Figure 4A). Moreover, SA treatment also inhibited the generation of H2O2 both in leaves and roots, which was consistent with the enhancement of CAT and APX activities (Figure 3A and Figure 4B,C). Ascorbic acid-glutathione (AsA-GSH) system comprises AsA, GR, and APX, converting H2O2 to H2O and MDA (Apel and Hirt, 2004). It has been established that SA can stimulate AsA-GSH cycle to increase the efficiency of antioxidants. Our study revealed that APX and GR activities were increased, leading to decreased H2O2 and MDA contents (Figure 3A, C and Figure 4C,D).

The secondary phase of *P. brassicae* lifecycle is associated with cell division and cell elongation, leading to gall formation (Lemarié et al., 2015). Auxin is an essential hormone that plays key roles in root development, including gall formation(Ludwig-Müller, 2014). Auxin level, as well as the expression of hormone - related genes, was changed after inoculation with *P. brassicae* in *B.napus* (Prerostova et al., 2018). Previous research showed many pathogens positively regulate auxin biosynthesis or modulate auxin signaling to make hosts more susceptible to infection (Kidd et al., 2011; Kazan and Lyons, 2014). In *Arabidopsis*, it has been stablished that SA or its analogue treatment represses auxin level and signaling (Wang et al., 2007). Therefore, we hypothesized that the exogenous SA treatment may also affect auxin biosynthesis and signaling, thus decreasing *P. brassicae* infection abilities and increasing plant resistance to infection, which is deserved to be further studied.

**Conclusions**

Collectively, we conclude that *P. brassicae*-infected pakchoi over products ROS and MDA, resulting in membrane lipid peroxidation, and damaging cell structures and functions. Exogenous SA significantly increases gene expression levels and antioxidant enzyme activities to decrease ROS contents and reduce or prevent membrane lipid peroxidation, leading to increased clubroot resistance in pakchoi.

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**Conflict of interest**

All authors declare that they have no conflict of interest.

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