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SHORT COMMUNICATION

Induction of disease resistance by salicylic acid and calcium ion against *Botrytis cinerea* in tomato (*Lycopersicon esculentum*)

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ABSTRACT

This study evaluated effect of single salicylic acid (SA), calcium ion (Ca^{2+}) or their combination on disease resistance of tomato (*Lycopersicon esculentum* cv. L402) against fungal pathogen *Botrytis cinerea* (*B. cinerea*). Elicitation with single SA, Ca^{2+} or their combination, particularly combined treatment, caused a remarkable increase ($p<0.05$) in accumulation of hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-), phenylalanine ammonia-lyase (PAL) activity, expression level of *PR* (pathogenesis related protein) gene in tomato plants and resulted in elevating resistance against *B. cinerea*. These results suggested that SA and Ca^{2+} played important role in induction of disease resistance against *B. cinerea*. Therefore, SA and Ca^{2+} might have potential to control gray mold disease of tomato.

Keywords: Salicylic acid; Calcium ion; Tomato; *Botrytis cinerea*; Induced resistance

INTRODUCTION

Tomato is one of the most popular vegetables in the world and plays very important role in healthy and nutritious diet (Hu et al., 2014). However, fungal and bacterial diseases seriously threaten tomato crops. Grey mold, caused by fungal pathogen *B. cinerea*, is one of the most serious diseases of tomato plants (Złotek and Wójcik, 2014). The pathogen can infect tomato leaf, stem, flower and fruit and lead to severe production loss (Małolepsza, 2006). Thus, desired method for effectively controlling disease caused by *B. cinerea* is required.

Researchers find elicitation is a new alternative technique for plants protection in recent years (Małolepsza, 2006). Plants respond to invading pathogen through activating basic resistance mechanism to prevent diseases (Robert-Seilaniantz et al., 2011). This process is mainly organized by a complicated hormonal network including SA, abscisic acid (ABA), jasmonic acid (JA) and nitric oxide (NO). SA is usually regarded as major part of defense response against pathogen (Angulo et al., 2015). Studies find systematic resistance against *B. cinerea* in Arabidopsis is mainly dependent on production of SA and its responsiveness (Segarra et al., 2013). Besides, exogenous Ca^{2+} also involves activation of response against pathogen (Castañeda and Pérez, 1996; Ranty et al., 2012). In previous study, it has been reported Ca^{2+} can enhance tomato resistance against *B. cinerea* (Li et al., 2012). Nevertheless, effect of SA combined with Ca^{2+} on disease resistance of tomato plants against *B. cinerea* does not further investigate.

In current study, tomato plants were treated with single SA, Ca^{2+} or their combination, respectively. Concentrations of H_2O_2 and O_2^-, PAL activity and expression level of *PR* gene were investigated and mechanism for SA and Ca^{2+} induced defense response against *B. cinerea* was discussed.

MATERIALS AND METHODS

Growth condition of tomato plants

Growth condition of tomato plants was performed using method of Li et al. (2012). The cultivated tomato hybrid (*Lycopersicon esculentum* cv. L402) seeds were sown in planter box and sprouted at 85% relative humidity and 28°C. Ten-day-old seedlings were transplanted to

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jiffy pot containing the established substrate consisted of vermiculite and nutrient soil (1:2, v/v). All tomato plants were carefully grown at 25/15°C, photoperiod 14/10 hours day/night and 65% relative humidity in greenhouse for 4 weeks.

Growth condition of pathogen

Fungal pathogen *B. cinerea* was cultured on a potato-dextrose agar medium at 25°C and conidial spore suspension (10^6 mL^{-1}) was prepared from seven-days-old fungus culture using 2% glucose solution (Li et al., 2012).

Plants treatment and pathogen inoculation

Plants treatment and pathogen inoculation were carried out using method of Li et al. (2012). Five-leaf stage tomato plants were respectively sprayed with 2 mM SA, 8 mM $CaCl_2$ or combination of 2 mM SA and 8 mM $CaCl_2$ (15 mL per plant). The same tomato plants sprayed with distilled water were served as control. After treatment, plants were kept in growth chamber for 3 days as described above. The seedlings were then inoculated by spraying *B. cinerea* spore suspension (5 mL per plant). After 5 days, development of disease was assessed using method of Flors et al. (2007).

Assay of H_2O_2

H_2O_2 was measured using method of Capaldi and Taylor (1983). Tomato leaf was adequately ground in 5% trichloroacetic acid (5 mL g^{-1} leaf tissue) at 4°C and centrifuged for 15 minutes at 8,000 rpm at 4°C. Supernatant was neutralized to pH 3.6, and then mixed with 3.4 mM methylbenzothiazoline hydrazone. After that, mixture solution (15 mL) was blended with 25 mL of horseradish peroxidase solution (1 U mL^{-1}) in 0.2 M sodium acetate buffer (pH 3.6). After 2 minutes, reaction was terminated by adding 0.1 mL of 1 M HCl and then optical density at 630 nm was measured against a blank (0.2 M sodium acetate buffer) and compared with standard calibration curve of H_2O_2 standard (Sinopharm, China).

Assay of O_2^-

O_2^- was measured using method of Elstner and Heupel (1976). Each leaf sample (10 g) was adequately homogenized in 15 mL of 0.05 M phosphate buffer (pH 7.8). After centrifugation at 8,000 rpm for 15 minutes at 4°C, supernatant (1 mL) was blended with 1 mM hydroxylamine hydrochloride solution (0.5 mL) and 0.1 M phosphate buffer (0.5 mL) and maintained for 1 hour at 25°C. Then, mixture was mixed with 1 mL of 7 mM naphthylamine and 17 mM aminophenylsulfonic acid solution and maintained for 20 minutes at 25°C. Afterwards, absorbance value at 530 nm was determined. Na_2NO_2 was employed for standard calibration curve with a UV-2600 UV-visible spectrophotometer (Unico, USA).

Assay of PAL activity

Pretreatment of sample was performed using method of Li et al. (2012). Leaf tissue (1 g) was adequately ground with 10 mL of 0.1 M boric acid-borax buffer (pH 8.8) at 4°C and centrifuged for 15 min at 8,000 rpm at 4°C to obtain supernatant (enzyme extract). PAL activity was measured using method of Dogbo et al. (2012). Reaction mixture was composed of 0.15 M phenylalanine solution (3 mL) and 1 mL of enzyme extract. It was incubated for 40 minutes at 37°C and optical density at 290 nm was determined. Protein was tested using method of Bradford (1976). Specific enzyme activity was presented as mmol cinnamic acid formed in enzyme reaction per hour per mg of protein (mmol h^{-1} mg^{-1} protein).

Assay of gene expression

Total RNA from tomato leaf was separated using RNAprep Pure Plant Total RNA Extraction kit (Qiagen, Germany). The cDNA was synthesized from total RNA (2 μg) with High Capacity cDNA Archive kit (Applied Biosystems, USA) and blended with 0.2 μM of primer and SYBR Green PCR Master Mix (Qiagen, Germany) as template. Real-time polymerase chain reaction (RT-PCR) analysis was carried out by ABI Prism 7500 Sequence Detection System and Software (PE Applied Biosystems, USA) (Jain et al., 2006). Tomato *Actin* gene was served as internal standard. Genes and primers used in the study were: *Actin* forward primer TGTCCCTATTTACGAGGGTTATGC, *Actin* reverse primer AGTTAAATCACGACCAGCAAGAT (GenBank accession number, Q96483); *PR* forward primer GGAAACTTCACTGTCAGACGTC, *PR* reverse primer GTGTCTCTGACACTTGTCGTCC (GenBank accession number, DQ159948).

Statistical analysis

All experiments were carried out in triplicate and statistical analysis was performed using SPSS statistical software package (SPSS, USA) (Angulo et al., 2015). All data were presented as mean ± standard deviation. Statistical significance was evaluated using one-way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) test of SPSS software. Difference at $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

SA and Ca^{2+} enhanced tomato resistance against *B. cinerea* infection

There are some reports that single SA or Ca^{2+} participates resistance acquisition of plants to prevent diseases (Angulo et al., 2015; Ei Oirdi et al., 2011; Li et al., 2012; Thuleau et al., 2013), but there are no data from studies about plants treated with combination of SA and Ca^{2+}. In present

study, the degree of disease development on tomato leaf was observably ($p<0.05$) reduced in tomato treated with single SA or Ca^{2+}. As shown in Table 1, SA or Ca^{2+} could cause a reduction in disease index by 46.95% and by 70.30% compared to control plant, respectively. However, combination of SA and Ca^{2+} was a more effective elicitor ($p<0.05$) compared to single SA or Ca^{2+}, which could cause a reduction in disease index by 38.50%. This might be because of production of stronger defense response against pathogens with combined treatment, which could effectively prevent pathogenic infection or reduce disease severity (Ranty et al., 2012; Thuleau et al., 2013). These results showed that SA and Ca^{2+} combination could better induce disease resistance of tomato plant against pathogen.

SA and Ca^{2+} induced accumulation of H_2O_2 and O_2^-

H_2O_2 and O_2^- play major roles in plant-pathogen interaction (Imada et al., 2015). Oxidative burst caused by H_2O_2 and O_2^- can continually kill pathogen and activate further defense reaction (Angulo et al., 2015). Therefore, more generation and accumulation of H_2O_2 and O_2^- in plant was associated with higher resistance to pathogen infection. Plant treated with single SA or Ca^{2+} caused the increase of H_2O_2 after 1 day of elicitation (Fig. 1). Similarly, treatment with combination of SA and Ca^{2+} also resulted in a prominent increase ($p<0.05$) in H_2O_2generation, as compared to control plant. H_2O_2 concentration in tomato leaf with combined treatment was dramatically higher ($p<0.05$) than those treated with single SA or Ca^{2+}. As shown in Fig. 2, the maximal O_2^- production rate of 0.45 nmol min^{-1} g^{-1} fresh weight (FW) on 2 days of elicitation was observed in plant treated with combination of SA and Ca^{2+}. Some studies showed H_2O_2 and O_2^- could regulate biotic stress in plants (Laloi et al., 2004; Neill et al., 2002; Vlot et al., 2009) and inhibit conidial germination of fungi (Chen et al., 1993). In present study, plant treated with combination of SA and Ca^{2+} could obtain higher generation of H_2O_2 and O_2^-, suggesting that rapid accumulation of H_2O_2 and O_2^- in tomato plants might be a critical step in induction of disease resistance against pathogen.

SA and Ca^{2+} induced increase of PAL activity

Analysis results showed tomato plant treated with single SA, Ca^{2+} or their combination enhanced PAL activity, as compared to control plant (Fig. 3). The highest PAL activity were 2.15 U mg^{-1} protein in single SA-treated tomato, 1.36 U mg^{-1} protein in single Ca^{2+}-treated tomato and 2.37 U mg^{-1} protein in tomato plant treated with combination of SA and Ca^{2+}, respectively. The data obtained in experiment indicated that PAL activity was inducted in tomato plants after SA and Ca^{2+} elicitation. This result was similar to that reported by Wang et al. (2011). These findings suggested higher PAL activity could be inducted when tomato plant was treated with combination of SA and Ca^{2+}.

Table 1: Disease index in tomato plants with different induced treatments

Index	Induced treatments			
	CK	SA	Ca	SA+Ca
Disease index (%)	74.86±2.19[a]	46.95±1.38[c]	66.30±2.07[b]	38.51±1.13[d]

CK: Control plant; SA: plant elicited with single 2 mM SA; Ca: plant elicited with single 8 mM $CaCl_2$; SA+Ca: plant elicited with combination of 2 mM SA and 8 mM $CaCl_2$. Different superscript letter indicates significant difference at $P<0.05$

SA and Ca^{2+} enhanced *PR* gene expression

It has been known some molecules such as ABA, JA and NO, are closely related to immune responses of plants, including induction of *PR* gene (Imada et al., 2015). There is a possibility that SA and Ca^{2+} directly act on tomato cell, resulting in variation in expression of *PR* gene involved in

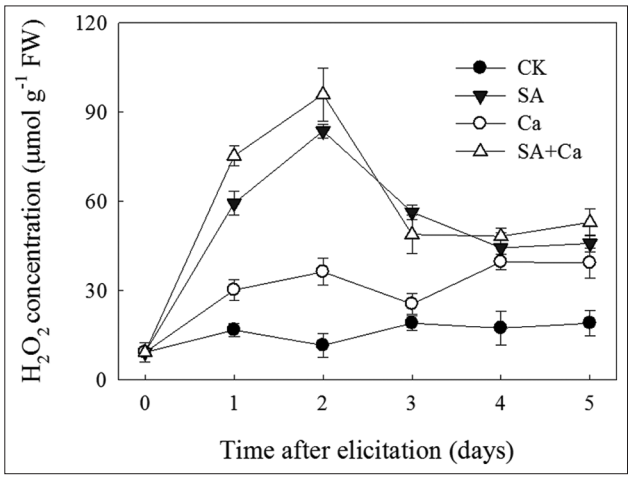

Fig 1. H_2O_2 concentration in tomato plants with different induced treatments. CK: Control plant; SA: Plant elicited with single 2 mM SA; Ca: Plant elicited with single 8 mM $CaCl_2$, SA+Ca; plant elicited with combination of 2 mM SA and 8 mM $CaCl_2$.

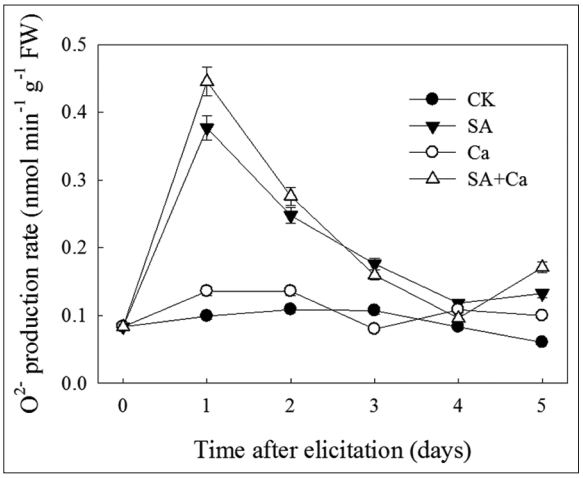

Fig 2. O^{2-} production rate in tomato plants with different induced treatments. CK: Control plant; SA: Plant elicited with single 2 mM SA; Ca: Plant elicited with single 8 mM $CaCl_2$, SA+Ca; plant elicited with combination of 2 mM SA and 8 mM $CaCl_2$.

pathogenesis related protein synthesis (Diaz et al., 2002). RT-PCR analysis showed that there was a obvious increase ($p<0.05$) in expression level of *PR* gene in the tomato leaf treated with single SA, Ca^{2+} or their combination, and reached peak values of relative expression level of 3.87-, 1.95- and 4.66-fold compared with control plant on 2 days after elicitation, respectively (Table 2). This revealed that *RP* gene of tomato plant treated with combination of SA and Ca^{2+} was rapidly induced at a relatively high expression level compared to single SA or Ca^{2+} treatment. Angulo et al. (2015) found that pathogenesis related gene expression obviously induced by SA against *B. cinerea* in tomato. Meanwhile, Ca^{2+} was acknowledged to increase expression level of pathogenesis related gene during induction of plant defense responses to pathogen (Thuleau et al., 2013). In present study, expression level of *RP* gene in tomato plant treated with combination of SA and Ca^{2+} was higher ($p<0.05$) than that treated with single SA or Ca^{2+}. This combined treatment might have greater potential to suppress development of gray mold when tomato leaves were attacked by *B. cinerea*.

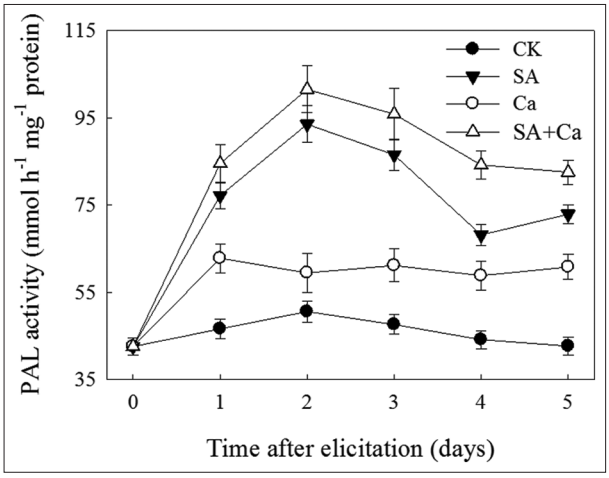

Fig 3. PAL activity n tomato plants with different induced treatments. CK: Control plant; SA: Plant elicited with single 2 mM SA; Ca: Plant elicited with single 8 mM $CaCl_2$; SA+Ca: Plant elicited with combination of 2 mM SA and 8 mM $CaCl_2$.

Table 2: Expression level of *PR* gene in tomato plants with different induced treatments

Time after elicitation (days)	Induced treatments			
	CK	SA	Ca	SA+Ca
0	1.00±0.14			
1	0.89±0.06^{c}	3.69±0.31^{a}	1.25±0.09^{b}	3.87±0.27^{a}
2	0.73±0.04^{a}	3.95±0.28^{a}	1.87±0.13^{a}	4.67±0.33^{a}
3	1.05±0.06^{d}	2.77±0.19^{b}	2.10±0.15^{c}	3.19±0.22^{a}
4	0.92±0.06^{d}	1.80±0.13^{b}	1.36±0.10^{c}	2.27±0.16^{a}
5	1.02±0.06^{d}	1.77±0.12^{b}	1.34±0.09^{c}	2.26±0.16^{a}

CK: control plant; SA: plant elicited with single 2 mM SA; Ca: plant elicited with single 8 mM $CaCl_2$; SA+Ca: plant elicited with combination of 2 mM SA and 8 mM $CaCl_2$. Different superscript letter in the same row indicates significant difference at $P<0.05$

CONCLUSION

Elicitation with single SA, Ca^{2+} or their combination caused a significant increase in accumulation of H_2O_2 and O_2^-, PAL activity, expression level of *PR* gene in tomato leaf and resulted in elevated resistance of tomato against *B. cinerea*. Therefore, SA and Ca^{2+} might have potential to control gray mold disease of tomato.

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Author contributions

Linlin Li performed experiment and analyzed result. Yu Zou designed experiment and wrote paper.

Abbreviations

SA: Salicylic acid; Ca^{2+}: Calcium ion; H_2O_2: Hydrogen peroxide; O_2^-: Superoxide anion; PAL: Phenylalanine ammonia-lyase; *PR*: Pathogenesis related protein; ABA: Abscisic acid; JA: Jasmonic acid; NO: Nitric oxide; RT-PCR: Real-time polymerase chain reaction; ANOVA: Analysis of variance; LSD:, least significant difference; FW: Fresh weight.

REFERENCES

Angulo, C., M. O. Leyva, I. Finiti, J. López-Cruz, E. Fernández-Crespo, P. García-Agustín and C. González-Bosch. 2015. Role of dioxygenase α-DOX2 and SA in basal response and in hexanoic acid-induced resistance of tomato (*Solanum lycopersicum*) plants against *Botrytis cinerea*. J. Plant Physiol. 175: 163-173.

Bradford, M. M. 1976. A dye binding assay for protein. Anal. Biochem. 72: 248-254.

Capaldi, D. J. and K. E. Taylor. 1983. A new peroxidase colour reaction: Oxidative coupling of 3-methyl-2-benzothiazolinone hydrazone (MBTH) with its formaldehyde azine application to glucose and choline oxidases. Anal. Biochem. 129: 329-336.

Castañeda, P. and L. M. Pérez. 1996. Calcium ions promote the response of *Citrus Limon* against fungal elicitors or wounding. Phytochemistry. 42: 595-598.

Chen, Z., H. Silva and D. F. Klessig. 1993. Active oxygen species in t he induction of plant systemic acquired resistance by salicylic acid. Science. 262: 1883-1886.

Dogbo, D. O., S. J. Gogbeu, B. n'Zue, K. A. Yao, G. P. Zohouri, J. A. Mamyrbekovabekro and Y. A. Bekro. 2012. Comparative activities of phenylalanine ammonia-lyase and tyrosine ammonia-lyase and phenolic compounds accumulated in cassava elicited cell. Afr. Crop Sci. J. 20: 85-94.

Diaz, J., A. T. Have and J. A. Van Kan. 2002. The role of ethylene and wound signaling in resistance of tomato to *Botrytis cinerea*. Plant Physiol. 129: 1341-1351.

El Oirdi, M., T. A. El Rahman, L. Rigano, A. El Hadrami, M. C. Rodriguez, F. Daayf, A. Vojnov and K. Bouarab. 2011. *Botrytis*

cinerea manipulates the antagonistic effects between immune pathways to promote disease development in tomato. Plant Cell. 23: 2405-2421.

Elstner, E. F. and A. Heupel. 1976. Inhibition of nitrite formation from hydroxylammoniumchloride: A simple assay for superoxide dismutase. Anal. Biochem. 70: 616-620.

Flors, V., M. L. Leyva, B. Vicedo, I. Finiti, M. D. Real, P. García-Agustín, A. B. Bennett and C. González-Bosch. 2007. Absence of the endo-β-1,4-glucanases Cel1 and Cel2 reduces susceptibility to *Botrytis cinerea* in tomato. Plant J. 52: 1027-1040.

Hu, G., J. Fan, Z. Xian, W. Huang, D. Lin and Z. Li. 2014. Over expression of SIREV alters the development of the flower pedicel abscission zone and fruit formation in tomato. Plant Sci. 229: 86-95.

Imada, K., S. Tanaka, Y. Masuda, T. Maekawa and S. Ito. 2015. Induction of disease resistance against *Botrytis cinerea* in tomato (*Solanum lycopersicum* L.) by using electrostatic atomized water particles. Physiol. Mol. Plant Pathol. 89: 1-7.

Jain, M., N. Kaur, A. K. Tyagi and J. P. Khurana. 2006. The auxin responsive GH3 gene family in rice (*Oryza sativa*). Funct. Integr. Genomics. 6: 36-46.

Laloi, C., K. Apel and A. Danon. 2004. Reactive oxygen signaling: The latest news. Curr. Opin. Plant Biol. 7: 323-328.

Li, L., T. Li, X. Tao, M. Qi, Z. Yu and K. Zhang. 2012. Calcium promotes tomato (*Lycopersicon esculentum* Mill.) resistance to *Botrytis cinerea* by enhancing salicylic acid (SA) biosynthesis. Afr. J. Biotechnol. 11: 9013-9022.

Małolepsza, U. 2006. Induction of disease resistance by acibenzolar-S-methyl and o-hydroxyethylorutin against. *Botrytis cinerea* in tomato plants. Crop Prot. 25: 956-962.

Neill, S., R. Desikan and J. Hancock. 2002. Hydrogen peroxide signaling. Curr. Opin. Plant Biol. 5: 388-395.

Ranty, B., V. Cotelle, J. P. Galaud and C. Mazars. 2012. Nuclear calcium signaling and its involvement in transcriptional regulation in plants. Adv. Exp. Med. Biol. 740: 1123-1143.

Robert-Seilaniantz, A., M. Grant and J. D. G. Jones. 2011. Hormone crosstalk in plant disease and defense: More than just jasmonate-salicylate antagonism. Annu. Rev. Phytopathol. 49: 317-343.

Segarra, G., G. Elena and I. Trillas. 2013. Systemic resistance against *Botrytis cinerea* in *Arabidopsis* triggered by an olive marc compost substrate requires functional SA signaling. Physiol. Mol. Plant Pathol. 82: 46-50.

Thuleau, P., D. Aldon, V. Cotelle, C. Brière, B. Ranty, J. P. Galaud and C. Mazars. 2013. Relationships between calcium and sphingolipid-dependent signaling pathways during the early steps of plant–pathogen interactions. Biochim. Biophys. Acta. 1833: 1590-1594.

Vlot, A. C., D. A. Dempsey and D. F. Klessig. 2009. Salicylic acid, a multifaceted hormone to combat disease. Annu. Rev. Phytopathol. 47: 177-206.

Wang, A. Y., B. G. Lou, T. Xu and C. Lin. 2011. Defense responses in tomato fruit induced by oligandrin against *Botrytis cinerea*. Afr. J. Biotechnol. 10: 4596-4601.

Złotek, U. and W. Wójcik. 2014. Effect of arachidonic acid elicitation on lettuce resistance towards *Botrytis cinerea*. Sci. Hortic. 179: 16-20.