Influence of Genotype, Salinity, Sulfur Treatments and Planting Container Size on Growth, Yield and Incidence of Gray Mold in Broccoli Plants with Propolis Extract as Disease Control Treatment

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Abstract: A pot experiment was conducted at the Experimental Research Farm, Faculty of Agriculture, Suez Canal University, Ismailia in Fall 2013 till Spring 2014. The experiment included two broccoli genotypes ("Sultan" and "Marathon"), two levels of salinity treatments (0, 100 mM NaCl), two levels of sulfur (0, 3 g/L soil) and two different soil volume containers (2, 4 L), in split-split plot design. The objective was to investigate the impact of genotype, salinity, sulfur treatments and container size on plant growth, yield and incidence of gray mold in broccoli. In addition, the effect of propolis extract as a natural mean of disease control was also explored. The results of the experiment revealed that broccoli genotypes differed in their growth and yield response. Also, salinity treatment adversely affected the growth and yield of broccoli in both genotypes and sulfur treatments were not able to mitigate the unfavorable effects of salinity on broccoli plants. As a result of this experiment, gray mold in broccoli was reported for the first time in Egypt and the fungus was identified as *Botrytis cinerea* based on mycological characteristics. Broccoli genotypes showed different disease severity as "Marathon" cv. was highly susceptible, while "Sultan" cv. showed higher degree of resistance. RAPD analysis identified some specific DNA fragments discriminating between the two genotypes which can explain the different response of both genotypes for yield and disease incidence. Salinity treatment significantly increased the disease severity by an average of 15.6% and 21.2% when compared to the control for plants grown in large and in small culture container, respectively, which demonstrate the effect of container size on the disease response as the larger size promoted the disease severity. Sulfur application was the most effective treatment in decreasing disease severity by 100% in both genotypes and in both container sizes. In presence of salinity, the inhibitory effect of sulfur sustained in "Sultan" cv., while sulfur decreased the disease severity in "Marathon" cv. only by 52.5%. In addition, propolis extract displayed inhibitory effect on *Botrytis cinerea* growth in both genotypes. Overall, genotypic differences observed for yield and salinity tolerance suggest that breeding programs to enhance such important traits are feasible. Soil-supplied sulfur enhanced broccoli defense to disease and can be suggested as mean of managing nutrition to control plant diseases. Finally, propolis extract can be suggested as a natural mean of gray mold disease control in broccoli.

Keywords: *Botrytis cinerea*, gray mold, *Brassica oleracea* var. *italica*, salinity, propolis extract, sulfur, container size

INTRODUCTION

Broccoli (*Brassica oleracea* var. *italica*) is a cruciferous crop that is an excellent source of vitamin C, calcium, magnesium, carotenoids, and fiber (Kurilich *et al.,* 1999). Broccoli has received particular attention as the best source of glucosinolates, sulfur-containing compounds, which have been shown to possess health benefits (Fahey *et al.,* 2002). Glucosinolate hydrolysis products could help prevent cancer by enhancing the elimination of carcinogens before they can damage DNA and high intakes of cruciferous vegetables including broccoli have been associated with lower risk of cancer in some epidemiological studies (van Poppel *et al.,* 1999).

Genotypes of plants vary widely in their qualitative and quantitative traits, however, visible plant morphological variation is known to occur at a much lower frequency than at the DNA level (Evans *et al.,* 1984) and as a result, it is necessary to examine for potential variation at the molecular level (Donini *et al.,* 2000). Molecular techniques are valuable tools used in the analysis of genetic materials. Random amplified polymorphic DNA (RAPD) markers provide an efficient technique for polymorphism that allows rapid identification of specific DNA fragments. The use of RAPD markers is especially beneficial to discriminate between materials that are genetically similar and to evaluate genetic variability (Bernardo Royo and Itoiz 2004). Identically banding patterns (monomorphism) of the RAPD profiles obtaining from different genotypes suggest similar characteristics while bands unique to one genotype (polymorphism) demonstrate genetic variation. RAPD markers were used to discriminate among cultivars of cabbage (Cansian and Echeverrigaray, 2000), broccoli (Hale *et al.,* 2006), broccoli and cauliflower (Hu and Quiros, 1991), and oilseed rape (Lee *et al.,* 1996).

When a RAPD marker show complete linkage to resistance gene, then the polymorphic RAPD fragment should be cloned and sequenced, followed by designing and synthesizing specific primers that produce an amplification product only in the resistant plants. This specific marker allows a reliable and rapid germplasm screening for resistance in breeding programs to produce elite genotypes (Michelmore, 1995; Waugh, 1997). RAPD was successfully used to develop markers linked to downy mildew resistance in broccoli (Agnola *et al.,* 2000; Farinhó *et al.,* 2000; Giovannelli *et al.,* 2002).

Soil fertility is adversely affected by salinity which has emerged as one of the most serious factors

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limiting plant growth and productivity (Türkan and Demiral, 2009). The loss in plant productivity due to salinity is a consequence of imbalance in ionic and nutrients contents concentration and osmotic effects (Ashraf, 2009) resulting in the production of reactive oxygen species (ROS). Any adaptation that regulates ROS generation in plants will provide efficient defense mechanism for salinity tolerance (Hasegawa *et al.,* 2000; Munns and Tester, 2008). Sulfur is increasingly being recognized to have potential in modulating salinity stress response (Nazar *et al.,* 2011).

Recently, the role of sulfur in plant nutrition has changed and currently sulfur has become one of the most limiting nutrients in agricultural products (Eriksen *et al.,* 2004). Nitrogen and sulfur are the only two fertilizer elements that are constituents of amino acids and subsequently proteins and enzymes. Biswas and Tewatia (1991) indicated that most crop species have higher yield and better quality products when there is an abundant amount of sulfur available in the soil. Elwan and Abd El-Hamed (2011) indicated that sulfur-treated broccoli plants produced higher yield comparing to non-treated plants and the response was more pronounced in genotype "Marathon" than genotype "Sultan". Plants assimilate inorganic sulfur into methionine (Nicoforova *et al.,* 2003), the precursor for glucosinolate production (Moreno *et al.,* 2006). The importance of an adequate supply of sulfur for glucosinolate synthesis has been well documented (Mailer, 1989; Kim *et al.,* 2002). Many of the breakdown glucosinolate products have been implicated in the interaction between *Brassica* plants and their pests and pathogens due to their role in defense mechanisms (Mithen, 1992; Rosa *et al.,* 1997).

Varying container size alters the root volume of the plants, which can significantly influence plant growth. The main effect of decreased container size is that it increases root restricting conditions (NeSmith and Duval, 1998). Plants experience many physiological and morphological changes in response to reduced rooting volume. Root restriction and container size can impact root and shoot growth, biomass accumulation, photosynthesis, leaf chlorophyll content, flowering and yield. Reduced plant biomass under root restricting conditions could possibly be due to a lower photosynthetic rate (Ruff *et al.,* 1987).

The fungicidal effect of foliar-applied sulfur has been exploited long time ago. In comparison, the significance of soil-applied sulfur for disease resistance only became evident recently. Elemental sulfur has been used efficiently against infections of grapes by powdery mildew (*Uncinula necator*). In addition to the direct fungicidal effect of foliar-applied sulfur, there was a nutrient-based effect of soil-applied sulfur that resulted in a lower rate of leaf and grape infection and producing a significantly higher yield compared with controls (Bourbos *et al.,* 2000).

Botrytis cinerea and other *Botrytis* species are important pathogens of numerous plants including vegetables, orchard crops, ornamental plants and stored agricultural products (Jarvis, 1977; Elad *et al.,* 2007). *Botrytis cinerea* is a common fungus that damages leaves, flowers, stems, fruits and other parts of many plants (Ellis, 1971). Botrytis often attacks very new seedlings, especially if the soil is highly fertilized. It often starts on the leaves as brown spots or patches which change into a grayish furry mould (Williamson *et al.,* 2007). In particular, young plants keel over and die very quickly once affected. Conditions that encourage moulds are over-watering, over-crowding and over-feeding.

Propolis is a resinous hive product collected from plant buds by bees and its color range from green to dark brown according to plant source. Bee products such as honey, pollen and propolis are used for the treatment of several diseases. It is believed that propolis can be used as a perfect antibiotic agent (Cherbuliez, 1996; Feraboli, 1996; Grange and Davey, 1990; Schmidt and Schmidt, 1996). Investigations have indicated that propolis contains wax, flavonoids, amino acids, essential oils, pollen, minerals and organic matters (Walker and Crane, 1987; Crane, 1990; Scheller, 1990). There has been considerable emphasis on the studies involving propolis (Crane, 1990; Ghisalberti, 1979; Yamauchi *et al.,* 1992). It was established that flavonoids, benzoic acid, and derivatives found in propolis showed antibiotic, antiviral and antimycotic effect.

The objective of this study was to investigate the effect of salinity, sulfur treatment and container size on plant growth, yield and incidence of gray mold in two broccoli genotypes. Moreover, the influence of propolis extract as a natural mean of the disease control was also studied.

MATERIALS AND METHODS

Experiment set up

Seeds of broccoli were sown in 209-cell styrophom trays under greenhouse condition in late October 2013. Broccoli genotypes were "Sultan" (Asgrow Seed Company, USA) and "Marathon" (Sakata Seed America Inc., USA). The trays were filled with a mixture of peatmoss and vermiculite 1:1 (v/v) enriched with different nutrients. After emerging, they were watered with a commercial nutrient solution (19-19-19 N-P-K with micronutrients) at a dilution of 1:200. The seedlings were maintained under mean temperature of 29°C. The 5 weeks old transplants were moved to black plastic bags in early December 2013 and the experiment was terminated in mid March 2014. Half of the plants were planted in small size plastic bags (2 liter of soil) while the other half were planted in larger size bags (4 liter of soil). The soil was sandy soil (85.2% sand, 11.5% silt and 3.3% clay) with pH = 8.27 and electrical conductivity (EC) = 0.47 $dSm^{-}1$. Broccoli plants were treated with salty solution at the concentration of 0.0 and 100 mM NaCl twice weekly after one month of transplanting. Small culture bags were irrigated with 200 ml of nutrient solution (19-19-19 N-P-K with micronutrients) plus the salt solution while the larger bags were irrigated with 400 ml. Sulfur was applied to the soil at the rate of 3 grams for each liter of soil in the plastic bag and the amount was divided to 3 doses. The first dose was added after one month of transplanting while the second and third doses were applied after two weeks intervals.

The experiment was laid-out in split-split-split plot in randomized complete block design (RCBD) with three replicates. The experiment employed two genotypes ("Sultan" and "Marathon") in the main plots, two different soil volume containers (2 and 4 L) in the sub-plots, two levels of salinity treatments (0.0 and 100 mM NaCl) in split-split plots and two levels of sulfur (0 and 3 g/L soil) in split-split-split plots in an orthogonal fashion.

Fresh weight of curds was recorded on all plants in the experimental unit and then dried in forced-air oven at 70°C for 72 h to obtain the dry weight. Fresh weight of vegetative growth was recorded after the harvest of the curd of the plant. Both curd dry weight (%) and plant dry weight (%) were calculated according the following formula: [(Fresh Weight- Dry Weight)/Fresh Weight]x100.

Pathogenicity test

Pathogenicity of *Botrytis* sp. was tested on two broccoli genotypes "Sultan" and "Marathon". The two genotypes were planted in the winter of 2013. Young healthy leaves were excised, washed, and placed on moist paper towels in plastic trays (with 5 leaves per tray spaced at about 5 cm between leaves). Mycelial agar plugs (6 mm diameter) of *Botrytis* sp was removed from the colony margin of a 2-day-old PDA culture (18^{o} C), and one plugs was inoculated face down on each leaf. For the control treatment, one uncolonized PDA plugs were inoculated on each leaf. There were five trays (replicates) for each genotype. After incubation at 20^{o} C under the regime of 12-h light/12-h dark for 3 days, lesion length that developed at each inoculation site was measured after 24h. and 72h.

Disease severity

After 20 days from sowing, the young plants inoculated with (1x10^{6}) spores per ml by dropping 1 ml of inoculation on the young leaves according to Chen and Wang (2005). The plants were covered with plastic bags for one day to maintain enough moisture to induce the conidia to infect the plants. Disease severity index (DSI) was determined after 34 days from the sowing using the formula: DSI=∑(rating of each plant)/4 (number of plants rated) (Bussey and Stevenson, 1991) where, 0: no symptoms, 1: 1 - 24% leaf area infected, 2: 25 -49 % leaf area infected, 3: 50 -75 % leaf area infected and 4: 76 - 100 % leaf area infected.

Application of propolis extract against *Botrytis cinerea*

Propolis was collected from bee hives in Ismailia, Egypt in April 2014. Czapek - Dox agar was used as main medium throughout the study. The used propolis was extracted with hot water. Water extracts (0.1, 0.2 and 0.3%) of propolis were prepared. Medium was separately prepared in each water extract, after which a 120 ml of media were dispensed into 250 ml Erlenmeyer flasks and sterilized by autoclaving at 121° C for 15 min.

Assessment of inhibition of fungal growth

The effect of propolis at different concentrations (0.1, 0.2 and 0.3%) was determined against the growth of *B. cinerea*. Medium was dispensed into each Petri plates as 10 ml. Five mm. discs of the test fungi were cut from periphery of 7 days old cultures, inoculated upside down separately onto each assay plate and incubated at 20°C. The colony diameter was measured and mycelia inhibition percentage was calculated following Deans and Svoboda (1990). Three replicates of each treatment were similarly maintained and then averages have been calculated. Control sets were simultaneously run without using propolis. On the other hand, the effect of propolis at different concentrations (0.1, 0.2 and 0.3%) was determined against sclerotia germination of *B. cinerea*. The sclerotia were dipped in propolis of each concentration and the sclerotia were inoculated in the plates including the Czapek-Dox agar medium. Each plate included 3 sclerotia of the test fungi, and plates were incubated at 20°C. The percent of sclerotia germination was calculated. Ten replicates of each treatment were similarly maintained and averages have been calculated.

Percentage of Inhibition = (C-T/C) x100

***Where*;**

C: Colony diameter of mycelium from control Petri plate (mm.)

T: Colony diameter of mycelium from test Petri plate (mm.).

Genomic DNA isolation, PCR reaction and RAPD analysis

DNA of the two tested broccoli genotypes was extracted from the recent leaves of the plants as described by Murray and Thompson (1980). Small pieces (0.5g) of leaf tissue were frozen in liquid nitrogen in Eppendorf tubes and homogenized in 500 ml of extraction buffer (2% CTAB, 1.4 m NaCl, 20 mM EDTA pH 8.0, 100 mM Tris-HCl, pH 8.0, 0.1 m B-Mercato ethanol). The extract was incubated at 60 Cº for 20 min. to this, 500 ml of phenol : chloroform : isoamyl alcohol (24:24:1) were added and mixed by vortexing for 30 sec. followed by centrifugation at 10,000 g for 5 min. at room temperature.

The aqueous phase was transferred to another tube. This was once again extracted with 500 ml of chloroform: isoamyl alcohol (24:1) in Eppendorf tubes. To the aqueous phase, 0.6 volume of isopropanol was added, precipitated the genomic DNA and spooled the fibrous DNA. Genomic DNA was then washed three times with 70% ethanol, dried in vacuum, dissolved in TE containing 10 mg/ml RNase and incubated at 37 Cº for 30 min. followed by extraction with phenal: chloroform: isoamyl alcohol and the aqueous phase was transferred to a fresh tube. The genomic DNA was then precipitated by adding 0.3 M sodium acetate, pH 5.2 (final concentration) and 2.5 vol. of ethanol and collected by centrifugation at 10.000 x g for 20 min. at 4ºC. The pellet was washed with 70% ethanol, vacuum dried and dissolved in TE.

Ten random oligonucleotide (10 mer) primers (Laboratories of the Midland Certified Reagent Company Inc. Texas, USA) were tested for use in RAPD analysis and their sequences are presented in Table (1).

Table (1): The nucleotide sequence of primers used in the RAPD analysis

Primer	Nucleotide Sequence
A01	5'CAGGCCCTTC3'
A02	5'TGCCGAGCTG3'
A03	5'AGTCAGCCAC3'
A04	5'AATCGGGCTG3'
A05	5'AGGGGTCTTG3'
A06	5'GGTCCCTGAC3'
A07	5'GAAACGGGTG3'
A08	5'GTGACGTAGG3'
A09	5'GGGTAACGCC3'
A10	5'GTGATCGCAG3'

The PCR reaction were carried out in 50 µL volumes tubes containing 100 ng of genomic DNA, 10 µM of each primer, 200 µM of dATP, dDTP, dCTP, dGTP, 10 mMTris-HCL, pH 8.3, 50mM $MgCl_2$ and 0.001% gelatin. The Taq DNA polymerase (Promega, Corporation, Madison, WI) concentration was 1.5 units per assay. The PCR reaction was conducted using Eppendorf thermocycler programmed according to the following protocol that consisted of 1 min. at 95Cº followed by 55 cycles of 20 sec. at 94ºC, 30 sec. at 37ºC, and 2 min. at 72ºC as described by Nadig *et al.* (1998). Amplification products were electrophoresed in 1.5% Agarose gel in 1 x TAE buffer, stained with ethidium bromide and visualized with UV transilluminator and photographed- A1000 bp ladder (Promega, Corporation, Madison, WI) was loaded on the gel for calculation of fragment size.

Statistical analysis

The results were subjected to Duncan's multiple range test for comparing the sixteen treatment combination at a significance level (α= 0.05). Calculations were carried out using the software package Statistica™ for Windows version 6.1 (Statsoft Inc., 2001).

RESULTS AND DISCUSSION

Broccoli yield

Yield is the most important trait and the ultimate goal for production process. Increase in the crop yield can come from a variety of sources, such as increased genetic yield potential, improved agronomic practices, better adaptation to environmental conditions, greater resistance to pests and diseases and interactions between all these sources (Gifford and Evans, 1981).

The larger size culture bags showed higher performance in both plant and curd fresh and dry weight in both genotypes (Table 2). The reduction due to the smaller size container were 65.8% in "Sultan" cv. and 62.5% in "Marathon" cv. and this may be explained by the adverse effects of restricted culture container on plant growth and yield (White, 1980; Peterson *et al.,* 1991). The reduction was more pronounced in the smaller size container under salinity treatment compared with non-saline treatment (Table 2). This may be caused by the dilution effect of salty solution that occurs with larger size containers. Salinity treatment negatively affected the growth and yield of broccoli plants in both genotypes. The reduction in yield was 55.6% in "Sultan" cv. while the percentage reached 57.4% in "Marathon" cv. (Table 2).

Unexpectedly, in most cases sulfur treatments did not improve growth and yield neither in saline nor in non-saline treatments, except in non-saline treatment of "Marathon" cv. where the increase due to sulfur was pronounced (38.6%). This may be due to the low concentration of sulfur added to each treatment in current experiment (only 3 g per each liter of soil), and the positive effect of sulfur on broccoli yield described in the literature can be achieved by using sufficiently higher amount of soil-applied sulfur.

Sulfur is an essential nutrient for plants and plays an important role in their life. Sulfur deficiency inhibits the growth and development of plants and considerably reduces crop yields. Sulfur is a constituent of amino acids, such as methionine, cystine and cystein which are essential components of protein molecules. Sulfur is also present in vitamins, such as thiamine and biotin which play an important role in plant metabolism. Thiamine is a constituent of some enzymes catalyzing decarboxylation of organic and amino acids while biotin takes part in deamination and decarboxylation of certain amino acids. Consequently, sulfur plays a major role in carbohydrate and nitrogen metabolism in plants (Yagodin, 1984). In addition, sulfur is a component of glucosinolates, which are characteristics secondary metabolites in *Brassica* (Mithen *et al.,* 2000). The term Sulfur Induced Resistance (SIR) denotes the reinforcement of the natural resistance of plants to fungal pathogens by stimulating the metabolic processes involving sulfur through targeted sulfate-based and soil-applied fertilizer strategies (Haneklaus *et al*., 2006).

The amino acids cysteine and methionine are the major end-products of sulfate assimilation in plants. Cysteine is the basic compound for other sulfur metabolites such as glutathione (GSH), glucosinolates (GSL), phytoalexins, pathogenesis related (PR) proteins and H_2S. All of these compounds are linked to resistance mechanisms of plants. Cysteine is the first major sulfur compound, itself showing fungicidal effects (Vidhyasekaran, 2000). H_2S may be released prior to or after cysteine formation. Commonly, H_2S is regarded as being fungitoxic.

During the hypersensitive response of plants after infection with pathogens H_2O_2 is released rapidly, which modifies cell metabolism in favor of phytoalexin and PR-protein accumulation, and finally results in programmed cell death (Foyer and Rennenberg, 2000; Hammerschmidt and Nicholson, 2000). GSH seems to

be involved in cell wall reinforcement (Gullner and Komives, 2001) and is coupled to high ascorbic acid which is related to resistance against fungal pathogens (Vidhyasekaran, 2000). Phytoalexins as a means of induced disease resistance include PR-proteins, low-molecular weight antibiotics Glucosinolates are characteristic sulfur-containing secondary compounds of *Brassica* crops that act as phytoanticipins.

Sulfur-containing compounds play an important role in plant stress defense. Both glutathione and cysteine contents are found to increase under salt stress and glutathione may play a protective role against salinity stress in plants. Cysteine is incorporated into glutathione that is one of the major redox controllers and plays significant roles in scavenging ROS.

Table (2): Interaction effect of genotype, salinity, sulfur and container size on broccoli growth and yield

Genotype	Salinity (mM NaCl)	Sulfur (g/L soil)	Container Size (L)	Yield (g/plant)	Curd DW (g)	Curd DW (%)	Plant FW (g/plant)	Plant DW (g/plant)	Plant DW (%)
Sultan	0.0	0.0	4	64.72a*	10.64a	16.08	110.67bc	23.00ab	20.79
			2	26.84d	4.98bc	18.63	72.58ef	14.17c	19.46
			Average	45.78	7.72	17.36	91.63	18.59	20.22
		3.0	4	38.97bc	6.65b	17.04	113.07bc	20.17b	17.78
			2	14.01efg	2.81de	20.18	38.87gh	8.50de	22.01
			Average	26.49	4.73	18.61	75.97	14.34	19.90
		Average		36.14	6.23	17.99	83.80	16.46	20.06
	100	0.0	4	27.39d	5.07bcd	18.84	122.43b	19.00b	15.43
			2	5.76g	1.17e	20.33	67.80ef	10.25cd	15.14
			Average	16.58	3.12	19.57	85.12	14.63	15.29
		3.0	4	24.57de	4.73bcd	18.76	141.83a	23.33ab	16.27
			2	6.44g	1.46e	22.83	36.77gh	7.17e	19.52
			Average	15.51	3.10	20.8	89.90	15.24	17.90
		Average		16.05	3.11	20.19	92.21	14.94	16.60
	Average			26.10	4.67	19.09	88.01	15.70	18.33
Marathon	0.0	0.0	4	32.91cd	7.26b	22.08	80.30de	19.83b	24.89
			2	11.16fg	2.84de	25.28	52.33fgh	13.50cd	26.47
			Average	22.04	5.05	23.68	66.32	16.67	25.68
		3.0	4	49.52b	10.46a	21.26	103.52bc	26.50a	25.64
			2	22.29def	5.27bcd	23.62	54.07efg	12.83cd	23.73
			Average	35.93	7.87	22.44	78.80	19.67	24.69
		Average		28.97	6.48	23.06	7.56	18.17	25.19
	100	0.0	4	24.68de	5.88bc	23.85	70.53ef	11.60cde	16.48
			2	8.20g	1.88e	22.97	61.33ef	11.50cde	18.89
			Average	16.44	3.88	23.41	65.93	11.55	17.69
		3.0	4	13.07efg	3.40cde	26.03	94.55cd	14.17c	15.05
			2	3.45g	1.05e	30.21	31.43h	7.50e	23.85
			Average	8.26	2.23	28.12	62.97	10.84	19.45
		Average		12.35	3.05	25.77	64.45	11.19	18.57
	Average			20.66	4.77	24.41	68.50	29.36	21.88

*For each trait, means followed by the same letter are not significantly different at ($p \leq 5\%$)

The sulfur response genes involved in sulfate transport and assimilation or in related metabolisms induced under different stress conditions, suggesting the existence of a general adaptive response to an increased in the cell demand for reduced sulfur (Nikiforova *et al.,* 2003). These genes definitely were different in the two tested broccoli genotypes in the current study and this might explain the diverse performance between the two genotypes concerning disease infection. In addition, "Marathon" and "Sultan" cvs. have shown a significantly different performance regarding yield, nitrate and vitamin C content as well as response to sulfur application (Abd El-Hamed and Elwan, 2010, 2013; Elwan and Abd El-Hamed, 2011).

Further analysis was carried out to apply molecular tools to assess the polymorphism existed as well as discriminate between the two broccoli genotypes. Ten primers which produced good and reproducible polymorphic bands among the two genotypes have been used. Higher number of amplified bands is offering an excellent chance for detecting DNA polymorphisms among individuals (Williams *et al.,* 1990). The presence of unique bands indicates that each genotype had one or more novel sequences which were not found in the other genotype. Unique bands are very useful for genotype identification to differentiate specific genotype from others (Vishwanath *et al.,* 2010).

RAPD PCR analysis showed promising results concerning the decrementing between both genotypes. Primers showed high degree of polymorphism between the two genotypes (Figure 1). Several unique bands in only one genotype have been detected suggesting a high degree of genetic variation between the two genotypes. The analysis resulted in 14 unique bands in both genotypes out of the total 46 produced bands (Table 3) (Figure 1). "Sultan" cv. created 5 unique bands out of the total 37 produced bands while "Marathon" cv. created 9 unique bands out of the total 41 produced bands. Polymorphism level was different from one primer to the other. Primers A05, A07, and A08 were specific for "Sultan" cv., while primers A02 and A04 were specific to "Marathon" cv. Primer A07 produced the highest number of unique bands in "Sultan" cv. (3 bands), however, primer A04 gave the highest number of unique bands in "Marathon" cv. (7 bands) (Table 3) (Figure 1).

Table (3): Primers with arbitrary sequence tested for their effectiveness in the RAPD-PCR analysis that produced polymorphic bands in two broccoli genotypes

Primers	Sultan		Marathon		Polymorphism	
	No. of Bands	Unique Bands	No. of Bands	Unique Bands	No.	%
A01	5	0	5	0	0/5	0%
A02	0	0	2	2	2/2	100%
A03	6	0	6	0	0/6	0%
A04	1	0	8	7	7/8	87.5%
A05	1	1	0	0	1/1	100%
A06	8	0	8	0	0/8	0%
A07	7	1	6	0	1/7	14.3%
A08	3	3	0	0	3/3	100%
A09	6	0	6	0	0/6	0%
A10	0	0	0	0	0/0	0%
Total	**37**	**5**	**41**	**9**	**14/46**	**30.4%**

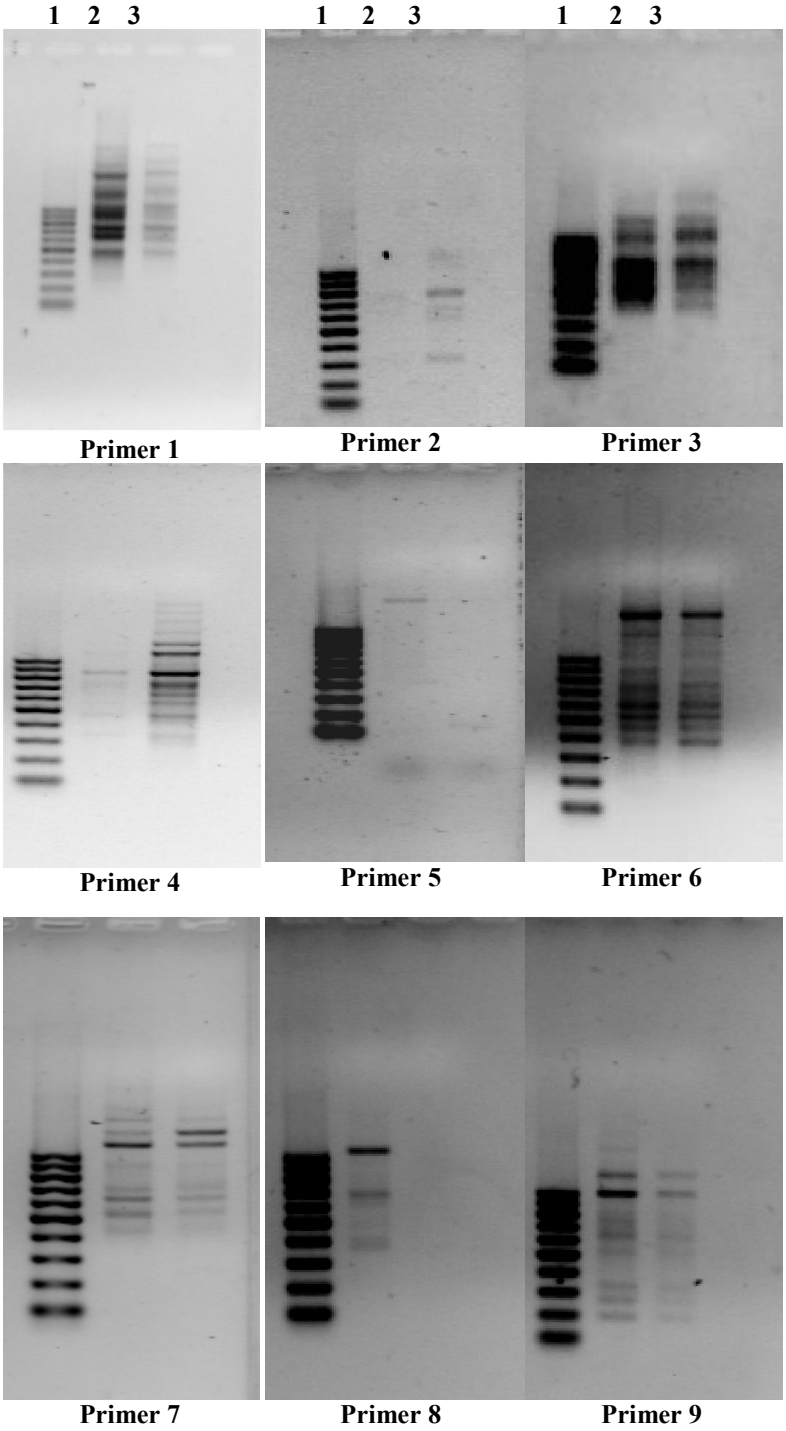

Figure (1): RAPD-PCR polymorphism of two broccoli genotypes using 10 primers (1- 1 kb DNA ladder; 2- "Sultan"; 3- "Marathon") (primer 10 was not shown)

Genotypes "Sultan" and "Marathon" showed considerably different performance regarding both gray mold and salinity tolerance. "Sultan" cv. was more resistant to grey mold compared to "Marathon" cv. and also was less affected by salinity. On the other hand, RAPD analysis identified number of unique bands to each genotype (Table 3) (Figure 1). Consequently, we may speculate that the specific bands identified in genotype "Sultan" are responsible for its tolerance to biotic and abiotic stresses exist in this experiment. Further genetic analysis might be able to show linkage between these specific marker sequence and tolerant genes (Avila *et al.,* 2003; Li and McVetty, 2013).

Tight linkage between molecular markers and genes for disease resistance can be of great benefit to disease resistance breeding programs by allowing following the DNA markers through the generations rather than waiting for phenotypic expression of the resistance genes. Once a gene has been found to be linked to markers, plants carrying the resistance gene can be easily identified (Lefebvre and Chèvre, 1995).

Interaction effect of genotype, salinity and sulfur treatment, container size on disease

As shown in Tables (4 and 5) and Figure (2), salinity treatment significantly increased the disease

severity (DS) value by an average of 15.6% as compared to the control for genotype "Marathon" grown in small containers and 21.2% for the same genotype grown in large containers. These results agree with Tzortzakis (2009) who reported that salinity induced severity of gray mold [*Botrytis cinerea* (De Bary) Whetzel] in lettuce (*Lactuca sativa* L. cvs. "Beta" and "Paris Island"). Cantrell and Linderman (2001) indicated that pre-inoculation of transplants with vesicular-arbuscular mycorrhizal fungi can help alleviate deleterious effects of saline soils on crop yield. Sulfur treatment was the most effective treatment in decreasing DS by 100% for both genotypes grown in small and large containers. The combination of salinity and sulfur treatment decreased DS by 100% for genotype "Sultan" grown in both container sizes when compared to the control, while it decreased the DS for genotype "Marathon" with an average of 52.5%.

Table (4): Pathogenicity test of *Botrytis cinerea* using leaves inoculation on genotypes "Marathon" and "Sultan"

Genotype	Lesion length (mm.)	
	24h.	72h.
Marathon	5.44	6.18
Sultan	0.11	0.11

Based on pathogenicity test, "Marathon" cv. was a highly susceptible genotype with 5.4 and 6.2 mm of lesion length after 24 and 72 hours, respectively. However, "Sultan" cv. was resistant genotype with lowest lesion length values of 0.11 mm (Table 4). Disease severity results showed also that "Marathon" cv. was highly susceptible genotype as compared to the "Sultan" cv. which was more resistant genotype. On the other hand, there was a relationship between the containers size and the severity of infection (the small containers showed high DS than that of large containers) and this may be due to the dilution effect of the salinity in large containers.

It is clear that sulfur treatment induced high plant resistance to grey mold in current study. Sulfur-induced resistance is the reinforcement of the natural resistance of plants against fungal pathogens through triggering the stimulation of metabolic processes involving sulfur. Sulfur is an essential macroelement for plant life and has numerous biological functions (Leustek *et al.,* 2000; Marschner, 1995). Soil-applied sulfur fertilization proved to significantly reduce infection rate and severity of fungal diseases. The potential efficacy of so-called Sulfur Induced Resistance (SIR) expressed as a reduction of the disease index was found to range from 5-50% and 17-35% in greenhouse and field, respectively (Haneklaus *et al.,* 2006). The metabolic pathway involved in SIR was suggested to involve the synthesis of phytoalexins, glutathione, glucosinolate and the release of sulfur-containing volatiles (Haneklaus *et al.,* 2006). Though the fungicidal effect of foliar applied elemental sulfur is known for a long time, it is a relatively new discovery that soil-applied sulfur in the form of sulfate can also have a positive effect on plant health (Haneklaus *et al.,* 2002).

Table (5): Interaction effect of genotype, salinity, sulfur treatment and container size on the control of grey mold in broccoli caused by *B. cinerea* under greenhouse conditions

Treatment	DS* (Small containers)		DS* (Large containers)	
	Marathon	Sultan	Marathon	Sultan
Salinity	3.26	0.48	2.52	0.38
Sulfur	0.00	0.00	0.00	0.00
Salinity – Sulfur	1.34	0.00	1.18	0.00
Control	2.82	0.44	2.08	0.28

DS= Disease Severity

Figure (2): Interaction effect of salinity and sulfur treatment on the control of grey mold in broccoli caused by *B. cinerea.*

It is suggested that salinity increase the DS due to the stress potential on the growing plants as reported in previous studies (Besri, 1993; Nachmias *et al.,* 1993). Salinity stress can increase disease severity through inhibition of host normal defense mechanism (Blaker and MacDonalds, 1986; MacDonalds, 1984). The physiological processes that are activated in plant tissues following pathogen invasion, which serve to limit pathogen establishment or spread, also may be impaired by salinity stress. Salinity also prevents the plant from absorbing water efficiently, forcing it to use more energy to hydrate itself and further weakening the plant. Poor water intake reduces shoot growth and causes diseases. The incorporation of sulfur into saline soil decreased the DS in both genotypes because of the major role of sulfur in control of foliar diseases as mentioned above.

Effect of propolis extract against *Botrytis cinerea*

The inhibitory effect of different concentrations of propolis extract was tested on the growth and sclerotia germination of *B. cinerea* (Tables 6 and 7). Propolis extract at concentrations of 0.1% and 0.2% had less effect on the mycelial growth of *B. cinerea* as the causal organism of grey mold in broccoli. However, all the concentrations showed inhibitory effect (about 31.63%) against *B. cinerea* until six days of incubation, inhibitory activity was partly increased on seven days of incubation. This increasing varied accordance to dose, effective material amount, effective level and fungus. However, the concentration of 0.3% of propolis showed higher inhibitory effect (81.83%) after 7 days of incubation in comparison with other concentrations. On the other hand, all propolis concentrations reduced the germination of the sclerotia, while the most effective concentration was 0.3% as it reduced the germination of sclerotia approximately (26.67%). Plant species used by bees as propolis source showed differences from region to region (Konig, 1985). Also, while propolis collected by bees grown in different regions showed similar properties due to some components, they showed highly different properties because of other components (Marletto, 1984). Only pinobanksin-3-acetate, 3-acetylpinobanksin, pinocembrin, p-coumaric acid and caffeic acid out of 26 or more components isolated propolis extract showed considerably antimycotinic effect (Ghisalberti, 1979). However, the same researcher reported that caffeic acid from propolis showed fungistatic effect against *Helminthosponum carbonum*. Lindenfelser (1967) reported that propolis inhibited the growth of 20 fungi among 39 isolated fungi samples. It was established that caffeic acid, benzyl cumarete, pinobanksin and pinocembrin found in propolis showed antimycotic properties. Yang *et al.* (2010) reported that Chinese propolis strongly inhibited mycelia growth and induced hyphae prominent abnormal morphological alterations of *Penicillium digitatum* and *Penicillium italicum* the causal organisms of green and blue mold of citrus, respectively. Also, Chinese propolis had strong detrimental effect on spore germination of the tested pathogens. Ozcan (1999) established that propolis known as balsam matter which collected by bees from several plants showed antifungal effect.

Table (6): The inhibitory effect of propolis extract against *Botrytis cinerea* the causal organism of broccoli grey mold

Days	Propolis Extract Concentration (%)	Inhibition Effect (%)
3	0.1	31.40
	0.2	45.84
	0.3	47.50
4	0.1	32.00
	0.2	46.23
	0.3	50.93
5	0.1	33.57
	0.2	48.64
	0.3	57.60
6	0.1	42.00
	0.2	49.97
	0.3	63.67
7	0.1	43.07
	0.2	63.73
	0.3	81.83

Table (7): Effect of propolis extract on the germination of sclerotia of *B. cinerea* in broccoli.

Propolis Extract Concentration % %	% Sclerotia Germination
0.0	100.0
0.1	86.67
0.2	63.34
0.3	26.67

CONCLUSION

Salinity treatment negatively affected the growth and yield of broccoli in both genotypes and sulfur treatments were not able to alleviate the adverse effects of salinity on broccoli plants. Resistance of broccoli to the gray mold was genotype dependent. Salinity treatment significantly increased the disease severest. Sulfur was the most effective treatment in decreasing the disease severity. Propolis extract showed inhibitory effect on infection with *Botrytis* in broccoli and can be recommend as an environment-friendly mean of disease control. For promoting resistance mechanisms, sulfur supply which only covers metabolic needs, is apparently not sufficient. A constantly high plant available sulfur reserve in the soil might also be required to satisfy the enhanced sulfur demand for plant defense during infection by fungal pathogens. Genetic differences between the two broccoli genotypes were obvious by RAPD analysis.

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تأثير التركيب الوراثي، الملوحة، المعاملة بالكبريت وحجم وعاء الزراعة على النمو والمحصول والإصابة بالعفن الرمادي في نباتات البروكلي مع استخدام مستخلص صمغ النحل كوسيلة لمقاومة المرض

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[١]قسم البساتين- كلية الزراعة- جامعة قناة السويس- الإسماعيلية ٤١٥٢٢

[٢] قسم النبات الزراعي- كلية الزراعة- جامعة قناة السويس- الإسماعيلية ٤١٥٢٢

أجريت تجربة أصص في المزرعة التجريبية لكلية الزراعة، جامعة قناة السويس، الإسماعيلية في خريف ٢٠١٣ واستمرت حتى ربيع عام ٢٠١٤. وتضمنت التجربة اثنين من التراكيب الوراثية للبروكلي ("سلطان" و"ماراثون") ومستويين من معاملات الملوحة (٠ و ١٠٠ مل مول كلوريد صوديوم) ومستويين من الكبريت (٠و ٣ جم/لتر تربة) وحجمين مختلفين من أوعية الزراعة (الأصص) (٢و ٤ لتر تربة) في تصميم القطع المنشقة ثلاث مرات. الهدف من هذه التجربة هو دراسة تأثير كل من التراكيب الوراثية للبروكلي والملوحة والمعاملة بالكبريت وحجم وعاء الزراعة على النمو والمحصول والإصابة بالعفن الرمادي في البروكلي. بالإضافة إلى ذلك تم دراسة تأثير مستخلص صمغ النحل كوسيلة طبيعية لمكافحة المرض. بينت نتائج هذه التجربة أن التراكيب الوراثية للبروكلي اختلفت في استجابتها للنمو والمحصول. و قد أثرت معاملة الملوحة سلبا على نمو وإنتاجية البروكلي في كلا الصنفين ولم تكن المعاملة بالكبريت قادرة على التخفيف من الآثار السلبية للملوحة على نباتات البروكلي. سجل في هذه التجربة حدوث مرض العفن الرمادي في البروكلي الناجم عن فطر *Botrytis cinerea* لأول مرة في مصر، وتم التعرف على الفطر على أساس الخصائص الميكولوجية. وأظهرت التراكيب الوراثية للبروكلي درجات مختلفة من شدة الإصابة، حيث كان صنف "ماراثون" أكثر حساسية للإصابة، في حين أظهر الصنف "سلطان" درجة عالية من المقاومة للمرض. اظهر تحليل RAPD بعض قطع الحمض النووي (bands) المحددة التي تميز بين الصنفين والذي يمكن أن تفسر الاستجابة المختلفة لكل من الصنفين لحدوث المرض. أدت معاملة الملوحة إلى زيادة شدة المرض معنويا وذلك بمعدل ١٥.٦٪ و٢١.٢٪ بالمقارنة بمعاملة الكنترول وذلك في النباتات التي كانت نامية في الأصص الكبيرة والصغيرة على التوالي مما يظهر تأثير حجم أوعية الزراعة على الاستجابة للمرض حيث أن الحجم الأكبر لأصص الزراعة زاد من شدة المرض. كانت المعاملة بالكبريت هي الأكثر فعالية في خفض شدة المرض بنسبة ١٠٠٪ في كلا الصنفين وفي كلا حجمي الأصص. في وجود الملوحة، استمر التأثير المثبط للكبريت في الصنف "سلطان"، في حين انخفض تأثير المعاملة بالكبريت على شدة المرض في الصنف "ماراثون" إلى ٥٢.٥٪ فقط. بالإضافة إلى ذلك، فإن مستخلص صمغ النحل اظهر تأثيرا مثبطا بشكل خاص على نمو الفطر في كل من الصنفين. بصفة عامة، فإن الاختلافات الوراثية التي تم ملاحظتها لكل من المحصول وتحمل الملوحة تبين إمكانية تبنى برامج تربية لتحسين هذه الصفات الهامة. الكبريت المضاف ارضيا حسن مقاومة نباتات البروكلي أثناء العدوى بجراثيم الفطر ويمكن اقتراحه كوسيلة من وسائل تغذية النبات للسيطرة على الأمراض النباتية. وأخيرا فإن مستخلص صمغ النحل يمكن استخدامه كوسيلة طبيعية لمقاومة مرض العفن الرمادي في البروكلي.