THE WATER EXTRACT OF *Saragassum fusiforme* IS A POTENTIAL ELICITOR OF INDUCED RESISTANCE IN *SOLANUM LYCOPERSICUM* BUT NOT IN *Nicotiana benthamiana*

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ABSTRACT

Molecules called elicitors induce plants' defense responses.Elicitor -induced resistance rarely leads to complete pathogen control, but reduce lesion size and/or number instead. We investigated a novel elicitor extractedfrom the brown sea algae (*Saragassum fusiforme*), whichis sowed to induce reactive oxygen species production and protection against powdery mildew and late blight diseasesoftomato (*Solanum lycopersicum*, cultivar Lady First)plants. On the other hand, the studied elicitor did not induce reactive oxygen species production in *Nicotiana benthamiana* plants nor protected them against late blight disease .

Keywords: *Elicitor, Induced Resistance, Saragassum fusiforme, Solanum lycopersicum, Nicotiana benthamiana.*

INTRODUCTIO N

Plant pathogens are a major challenge of plant growth and development. They can seriously affect growth, production, and quality of agricultural crops. Plants exhibit various sorts of acquired resistance enabling them to overcome the invasion of the vast majority of potential pathogens (Jones and Takemoto, 2004, Muthamilarasan and Prasad, 2013). The major trends of specialists to protect plants are disease control and developing resistant varieties. Nonetheless, improved resistance of pathogens to pesticides and fungicides, environmental and health concerns related to agro-chemical and long and costly breeding programs are considered major obstacles limiting the traditional plant protection methods (Duvick, 2005, Lopes et al., 2012, De La Fuente, 2013, Nicholls and Altieri, 2013, Sierotzki and Scalliet, 2013). When interact with pathogens, plants activate defense reactions upon the recognition of small, structurally conserved motif molecules within many microbial species. These molecules are called microbe associated molecular patterns or pathogen associated molecular patterns (MAMPs or PAMPs) (Jones and Takemoto, 2004, Kouzai et al., 2013). Defense reactions lead to acquired resistance if they were sufficient and well-timed, or can be overcome by the attacking pathogens.

Elicitors are agents that are able to induce plant's resistance. The term 'elicitor' was originally used for compounds that induce accumulation of antimicrobial phytoalexins in plants,and is now commonly applied to agents stimulating any type of defense response (Keen and Bruegger, 1977). An increasingly growing interest of scientists to explore new elicitors of plant resistance and understanding the molecular and biochemical basis of their action, is aiming at fostering plant protection strategies. Elicitors were extracted from bacteria, fungi, oomycetes, sea algae and plants or even chemically synthesized (Nurnberger, 1999, Walters and Fountaine, 2009, Armana and Ul Qader, 2012).

Elicitor applications lead to resistance reactions including; the production of reactive oxygen species (ROS), reactive nitrogen species (RNS), production of phytoalexins, induction of hypersensitive reaction (HR), callose deposition, lignin accumulation and expression of resistance related genes (Doke and Tomiyama, 1980, Takemoto et al., 1999, Matsukawa et al., 2013, Monjil et al., 2013, Takeuchi et al., 2013). Elicitors can be species specific or nonspecific, and can induce resistance both locally and systemically. For example, pretreatment of oligogalacturonides produced by partial enzymatic degradation of the

cell wall of citrus fruit rinds on *Ara bidopsis thaliana* induced resistance reactions and the plants' resistance against *Botrytis cinerea* (Klarzynski et al., 2003, Suárez et al., 2013). The first chemical resistance activator, Probenazole, was registered in Japan as Oryzemate in 1975, and since then many other chemical and biological activators have been developed, including ASM, registered as Bion and Actigard (Syngenta), Milsana (*Reynoutria sacalinensis* extract; KHH BioScience), Elexa (chitosan; SafeScience) and Messenger (harpin protein; Plant Health Care) (Walters et al., 2013). In this work, the eliciting ability of a crude water extract of the brown sea algae (*Saragassum fusiforme*) was analysed for inducing ROS production in tomato (*Solanum lycopersicum*cv. Lady First) and *Nicotiana benthamiana* plants. In addition, the ability of the extract to induce the resistance of *S. lycopersicum*and*N. benthamiana* against oomycete and fungal pathogens was also evaluated.

MATER IA LS AND METHODS

Biological materials and growth conditions: Tomato plants (*S.lycopersicum* cv. Lady First) were germinated from seeds (Aisan Seed C., Kiyosu, Japan) at 25-28 °C and then grown at 20-23 °C under a photoperiod of 16 h light/8 h dark period in environmentally controlled growth cabinets. Seeds of *Nicotiana benthamiana* were provided by the Leaf Tobacco Research Center (Japan Tobacco Inc., Tokyo, Japan). *N. benthamiana* plants were grown at 23 °C and 70% humidity under a 16 h photoperiod and an 8 h dark period in environmentally controlled growth cabinets. The pathogenic isolate [*Phytophthora infestans* (Mont.) De Bary], race 1.2.3.4 was used in the research; collection of zoosporangia and induction of zoospore production from *P. infestans* was performed. Zoosporangia suspensions from the *P. infestans* isolates were prepared as follows; *P. infestans* isolates were sub-cultured on rye-media for 7–10 days, 20 ml of water were added to the surface of the *P. infestans* colonies, which were then rubbed with a cotton swab to release the zoosporangia, the zoosporangia suspension was then incubated at 10 °C for 3 hours for zoospores production. Tomato plants infected with the obligate biotroph *Oidium spp*. were provided by Aichi Agricultural Research Centre (Aichi, Japan) and kept at 23-25 °C under a photoperiod of 16 h light/8 h dark period in environmentally controlled growth cabinets.

Inoculation: For plant-Pathogen interaction tests; leaflets of *S.lycopersicum* and leaves of *N. benthamiana* plants were inoculated with 0.5 ml and 1 ml aliquots of *P. infestans* zoospore (10^5 zoospores/ml) suspension, respectively, and covered with lens papers. The inoculated plants were kept at high humidity at 20°C for 1 day, and moved then into 23 °C growth cabinet. *S. lycopersicum* plants were mixed with plants infected with *Oidium spp*, and the positions of the plants were changed randomly every two days to insure uniformity of exposure to the airborne pathogen. The inoculated leaves were observed on daily basis for monitoring disease symptoms.

Elicitor extraction and preparation: Brown sea algae (*S. fusiforme*) were steamed at a temperature of 120 °C and a pressure of 2.0 kg/ cm^2 for 60 minutes. The steam was trapped and cooled. The obtained solution was used as sea algal product (AP).

Measurement of O_2^- production: To measure the relative intensity of O_2^- generation,L-012-mediated chemiluminescence-based photon counting was developed. L-012 (Wako, Osaka, Japan) is a luminol derivative that is highly sensitive to O_2^-(Kobayashi et al., 2007). To detect the O_2^-production in *S.lycopersicum* and *N. benthamiana* leaves, 0.5 mM L-012 in 10 mM MOPS-KOH (pH 7.4) were infiltrated to the intercellular space through the abaxial surface of leaves using a syringe without a needle. Chemiluminescence was monitored continuously using a photon image processor equipped with a sensitive CCD camera in a dark chamber at 20 °C (Aquacosmos 2.5; Hamamatsu Photonics, Shizuoka, Japan), and quantified using the U7501 program (Hamamatsu Photonics).

R ESULTS AND DISCUSSIO N

Reactive Oxygen Species production activity

Signaling via reactive oxygen species (ROS) is widely regarded to be central to disease resistance in plants (Mehdy, 1994; Wojtaszek, 1997; Fobert and Despres, 2005; Torres et al., 2006). ROS is also involved in the pathogen-antagonist interaction in postharvest biocontrol systems (Liu et al., 2013). ROS was reported to demonstrate direct antifungal and antimicrobial activity during pathogen infection. In addition, ROS contribute to downstream biochemical pathways of resistance such as callose deposition, salicylic acid (SA) signaling and the expression of pathogenesis-related (PR) genes (Alvarez et al., 1998 and Vellosillo et al., 2010). The superoxide anion (O_2^-) is one of ROS that is activated in *S.lycopersicum*,*N. benthamiana* and other plants in response to pathogen infections and elicitor application (Mehdy, 1994 andMatsukawa et al., 2013). In this work, we used O_2^- as a marker of induced resistance in response to elicitor application. O_2^- producing activity of *S.lycopersicum* and *N. benthamiana* due to AP elicitor application was measured by using O_2^- unique luminous reagent L-012. Figure 1 shows noteworthy induction of O_2^- production in leaves treated with AP elicitor at 1% and 10% concentrations measured 90 minutes after elicitors' application. Figure 1 also shows that *S.lycopersicum* plants responded to algal elicitor applications in a dose respective manner. Quite the reverse, the model solanaceous plant, *N. benthamiana*, did not show induction of ROS in response to algal elicitor treatments. Algal extract was applied to *N. benthamiana* leaves at several concentrations, and none of them showed superoxide accumulation (Figure 4). Based on O_2^- induction in *S.lycopersicum* and *N. benthamiana*, it might be claimed that algal product is a species-specific elicitor; nevertheless, our results show ROS accumulation in addition to other resistance reactions on potato plants as a result of algal elicitor (data not shown) which destabilizes this hypothesis.

Protection against diseases

Induced resistance reactions of plants could lead to disease prevention or reduction. *P. infestans* is an economically important filamentous pathogen of potato. Moreover, it causes late blight disease on a range of solanaceous species including but not limited to tomato and *N. benthamiana* (Becktell et al., 2006, Chaparro-Garcia et al., 2011). We investigated the effects of preceding algal elicitor applications on late blight severity caused by *P. infestans* inoculated onto *S.lycopersicum* and *N. benthamiana* subsequently. Severity of late blight on *S.lycopersicum* plants was reduced by around 27% measured 12 dpi as a result of algal elicitor (Figure 2-a). The numbers of infected leaves (Figure 2-b) as well as disease severity (Figure 2-b and c) were reduced on tomato leaves previously sprayed with algal elicitor. On the other hand, applications of algal elicitor on *N. benthamiana* did not result in reduced pathogen infection. Late blight symptoms were developed similarly on *N. benthamiana* elicitor-treated and water-treated leaves (Figure 5). The diminished severity of late blight on tomato leaves could be the results of two possible factors; direct antifungal activity of the studied algal product, or induced resistance of tomato which lead to reduced pathogenicity. Depending on the fact that similar algal elicitor applications did not lead to reduced severity of late blight on *N. benthamiana*, we assume the first hypothesis is not valid. Moreover, algal elicitor showed no antifungal activity against *P. infestans* culture on ray medium (data not shown). On the second hand, algal elicitor induced the superoxide production and accumulation on treated *S.lycopersicum* leaves. O_2^- possessesantifungal activity through it toxicity, and is also involved in other resistance reaction that might lead to reduced disease development and partial plant protection. Together, our results indicate that the superoxide O_2^-, which is produced in *S.lycopersicum* due to algal elicitor applications, is involved in the induced resistance against *P. infestans*.

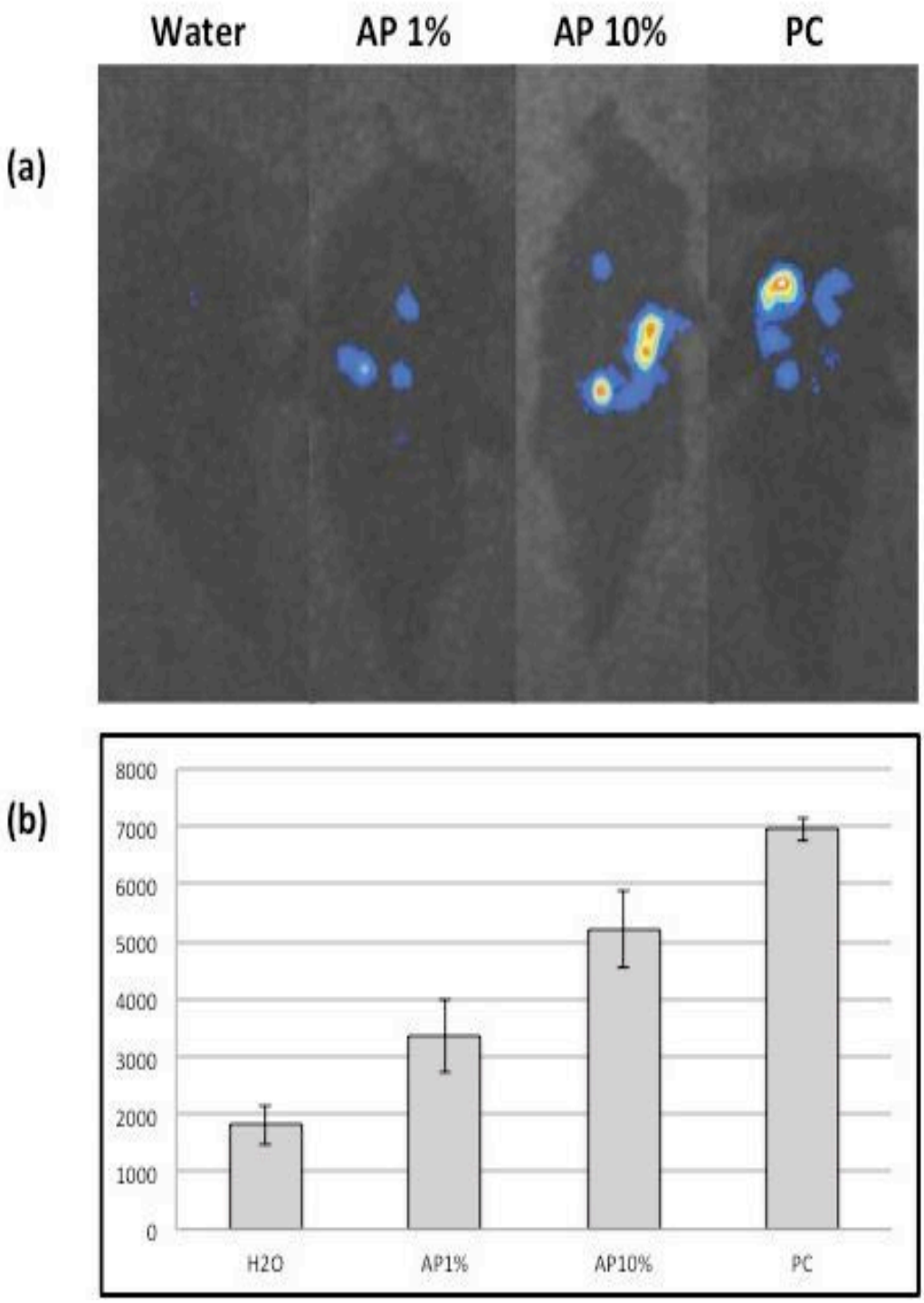

Fig. 1. Alga l Product (AP) induced O_2^- in tomato cv. Lady First (LF) plants. (a) *S. lycopersicum*cv. LF plants were sprayed with AP 1%, AP 10%, water (as a negative control) or hyphal wall components of *P. infestans* (as a positive control), and 90 min later the luminol derivative L-012 (0.5 mM L-012 in 10 mM MOPS-KOH (pH 7.4)) was infiltrated to the abaxial surface of the leaves using a needleless syringe . Chemiluminescence was monitored using a photon image processor equipped with a sensitive CCD camera in a dark chamber at 20 °C. Representative graphs were taken 90 mpt. (b) Data were quantified using the U7501 program. Shown data are the a verage of three repeated experiments.

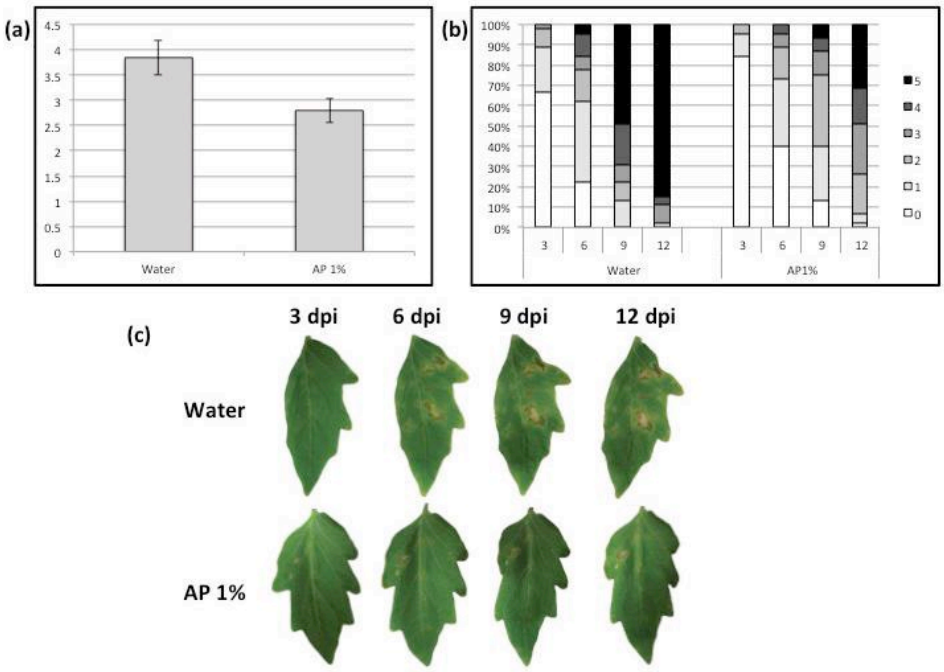

Fig. 2. Pla nt-pathogen (*S. lycopersicum*cv. Lady First-*P. infestans*) interaction in the presence of algal product (AP) elicitor. Tomato cv. LF plants were thoroughly sprayed with AP (1%) or water and inoculated with 0.5 ml of *P. infestans* (race 1.2.3.4) spores (10^6 spore/ml) 1 day later and kept in a humid chamber for another day, disease development was assessed daily from the 3rd until the 12th dpi. (a) Disease severity on *S. lycopersicum* leaves 7 dpi, for disease severity quantification, the pathogens' development on *S. lycopersicum* leaves was marked of 0 (if the leaf is not infected) to 5 (if the leaf is fully infected). (b) Percentage of infected leaves and disease severity of the AP- and water-treated plants from 3rd until 12th dpi.
(c) Representative leaves of both treatments photographed on the representative days of the experiment. Three plants were used in each experiment. Results are the average of three repeated experiments.

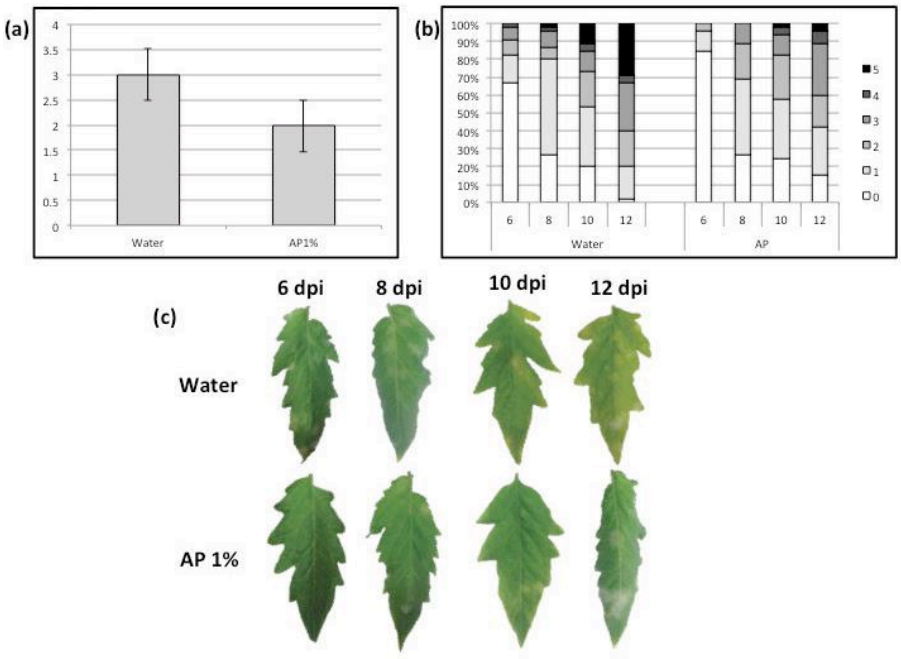

Fig. 3. Plant-pathogen (*S. lycopersicum*cv. Lady First-*Oidium spp.*) interaction in the presence of algal product (AP) elicitor. Tomato cv. LF plants were spray washed with AP (1%) or water and introduced into a growth chamber containing *Oidium spp.* infected plants 1 day later, disease development was assessed daily from the 6th until the 12th day after the introduction
(a) Disease severity on *S. lycopersicum* leaves 12 days after disease introduction, for disease severity quantification, the pathogens' development on *S. lycopersicum* leaves was marked of 0 (if the leaf is not infected) to 5 (if the leaf is fully infected). (b) Percentage of infected leaves and disease severity of the AP- and water-treated plants from 6th until 12th day after pathogen introduction. (c) Representative leaves of both treatments photographed on the representative days of the experiment. Three plants were used in each experiment. Results are the average of three repeated experiments.

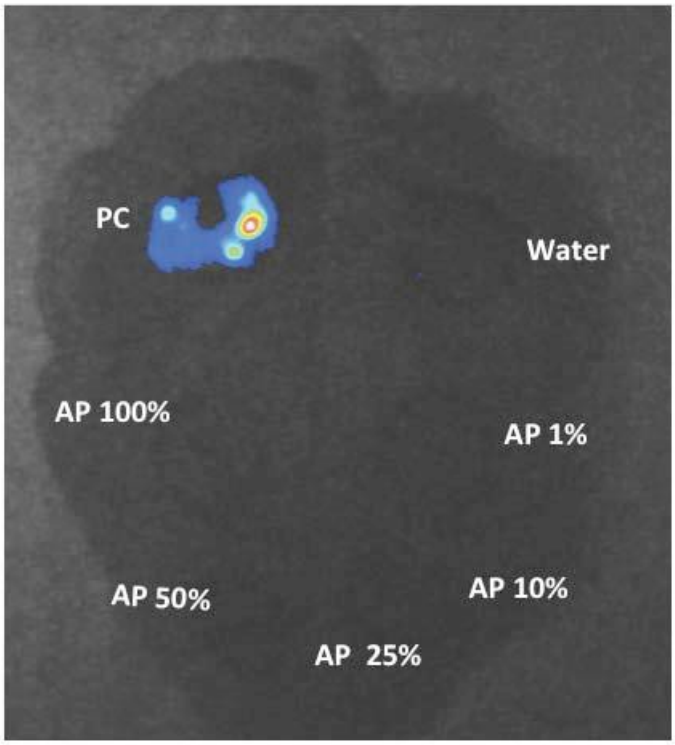

Fig. 4. Algal Product (AP) did not induced O^{-}_{2}production in *Nicot iana benthamina* plants. (a) *N. benthamina* plants were infiltrated through the abaxial leaf surface with AP 1%, AP 10%, AP 25%, AP 50%, AP 100%, water (as a negative control) or hyphal wall components of *P. infestans* (as a positive control), and 3 h later the luminol derivative L-012 (0.5 mM L-012 in 10 mM MOPS-KOH (pH 7.4)) was infiltrated to the abaxial surface of the leaves using a syringe without a needle . Chemiluminescence was monitored using a photon image processor equipped with a sensitive CCD camera in a dark chamber at 20 °C. Representative graph was taken 3 hpt.

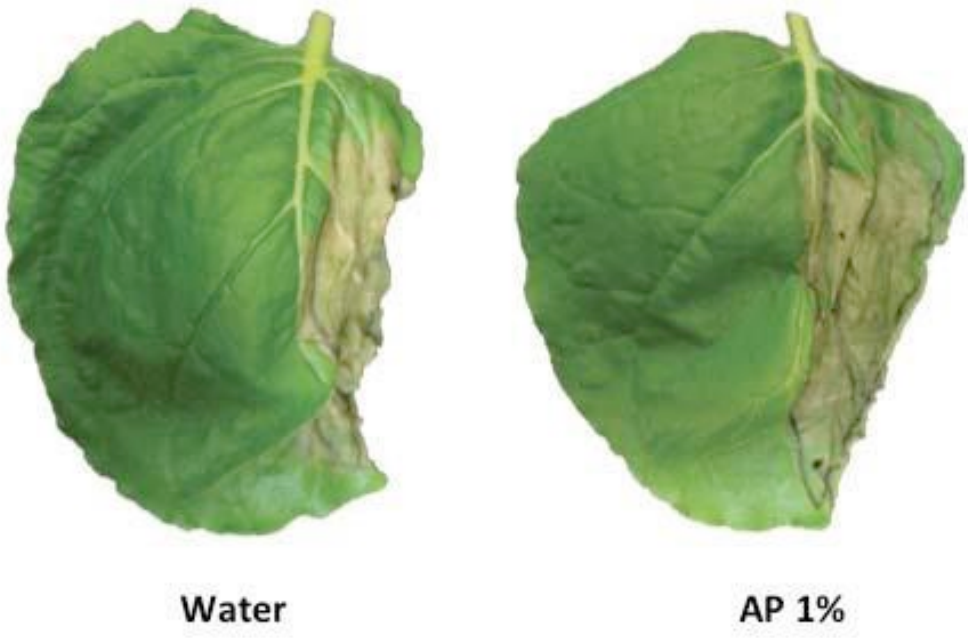

Fig. 5. Plant-pathogen (*N. benthamina-P. infestans*) interaction in the presence of algal product (AP) elicitor. *N. benthamina* plants were thoroughly sprayed with AP (1%) or water and inoculated with 1 ml of *P. infestans* (race 1.2.3.4) spores (10^6 spore/ml) 1 day later and kept in a humid chamber for another 24 h, disease development was assessed daily from the 2nd until the 12th day after inoculation. The experiment was repeated three times with similar results. Three plants were used in each experiment. Representative photographs were taken 7 dpi.

Following, we investigated whether algal elicitor prior applications lead to diminished-severity of powdery mildew disease on tomato plants. Elicitor-treated *S.lycopersicum* plants were introduced into a growth chamber containing other plants infected with the air born pathogen *Oidium spp*. Figure 3 shows that the severity of powdery mildew on *S.lycopersicum* plant leaves was noticed less in the case of algal elicitor-treated plants compared to the control. Prior applications of algal elicitor have lead to reduced average disease severity on treated plants. The largest protection was noticed at the end of our tests i.e. 12 days after disease introduction (Figure 3-a). Powdery mildew disease incidence and severity were delayed and reduced on algal elicitor treated plants(Figure 3-b and c). Obviously, we have no means of investigating the direct effect of algal elicitor on the obligate biotroph *Oidium* spp.

Powdery mildew and late blight are considered among the major diseases threatening tomato culture around the world. Tomato powdery mildew caused by *Oidium* spp. is a relatively newly reported disease. Kashimoto et al.(2003) reported susceptibility of all commercial tomato cultivars available in Japan to powdery mildew caused by *Oidium neolycopersici*. All the cultivar showed highest level of susceptibility. Matsuda et al. (2005) revealed fungicide-tolerant isolates of tomato powdery mildew on naturally infected tomato leaves, indicating the necessity of alternative measures to control the pathogen. Late blight is a devastating disease of tomato as well. Once an unprotected tomato crop (field, greenhouse, and/or plastic-cover cultures) is infected by *P. infestans*, the whole crop can be destroyed within 7 to 10 days (Fry, 2008). Economic losses may be in the form of reduced yield, lower quality of the fruit, diminished storability and increased cost associated with fungicide applications (Fontem et al., 1996). Alternative measures to control tomato powdery mildew and late blight seem vital. Induced resistance is an increasingly growing alternative method of plant protection. Our studied algal elicitor is able to induce resistance of tomato cv. Lady First plants leading to reduce powdery mildew and late blight severities. However, it is obvious that algal product treatment did not completely suppress infection and disease development. Kuc (1982) reported that elicitor-induced resistance rarely leads to complete pathogen control, but reduce lesion size and/ or number instead. However, reduced severity of the pathogen might lead to easier control. An elicitor added to certain fungicides, amplified the fungicides efficacy, which leaded to achieving the intended antifungal activity by applying highly reduced amount of fungicide (Bounatesta et al., 2013). Purification of algal elicitor as well as combinations with other plant protectants might show better control of tomato powdery mildew and late blight diseases.

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