

Effects of nano-TiO₂ semiconductor sol on prevention from plant diseases

Ping ZHANG, Haixin CUI*, Xiuli ZHONG, Lingling LI

*Institute of Environment and Sustainable Development in Agricultural, Chinese Academy of Agricultural Sciences,
Beijing 100081, P.R. China*

Received date: January 26, 2007

Abstract [Objective] The effects of nano-TiO₂ semiconductors sol (nano-TiO₂ sol) on plant diseases prevention were studied. **[Methods]** The antibacterial abilities of nano-TiO₂ sol were identified by using bacterial pathogens of *Pseudomonas syringae pv. lachrymans* and *Xanthomonas vesicatoria*. The effects of nano-TiO₂ sol on plant diseases prevention were measured by method of artificial inoculation experiment with cucumber plants inoculated with *Pseudomonas syringae pv. lachrymans* and field experiment with cucumber seedling naturally infected by *Pseudoperonospora cubensis*. **[Results]** Results showed that nano-TiO₂ sol possessed strong antibacterial power by forming continuous and stable antibacterial films on surface of substances. Artificial inoculation experiment and field experiment indicated that spraying cucumber leaves with certain concentration of nano-TiO₂ sol could significantly reduce lesion areas, disease incidences and disease indexes. **[Conclusion]** It was initially confirmed that nano-TiO₂ sol could effectively inhibit the development and pervasion of plant bacterial / fungal diseases.

Keywords: nanomaterial; TiO₂; sol; semiconductor; plant diseases; prevention

1 Introduction

Since the discovery of photoinduced water cleavage on titanium dioxide (TiO₂) electrodes by Fujishima and Honda in 1972^[1], TiO₂ photosemiconductor have attracted great attention as alternative materials to aid in the purification of water and air^[2, 3]. When illuminated under near-UV light, nano-TiO₂ semiconductor generates holes (h⁺) and hydroxyl radicals (OH·) in the valence band (VB), and electrons and superoxide ions (O₂⁻) in the conduction band (CB). The reactive oxygen intermediates (ROIs) generated by the TiO₂ photocatalytic reactions have strong oxidizing power, which can decompose and mineralize organic compounds through participating in a series of oxidation reactions and cause various damages to living organisms^[4-9]. In 1985, Matsunaga *et al* reported for the first time the microbiocidal effect of TiO₂ photocatalytic reactions^[5]. Since then, research work on TiO₂ photocatalytic killing has been intensively conducted on a wide spectrum of organisms including viruses, bacteria, fungi, algae, and cancer cells^[7-9]. Furthermore, it was also found by plant pathologists that

one of the earliest events in the hypersensitive response (HR), characterized by cell death, was a burst of oxidative metabolism leading to the generation of O₂⁻ and subsequent accumulation of H₂O₂^[10]. These ROIs may directly facilitate pathogen killing and rapidly drive oxidative cross-linking of the cell wall^[10]. Moreover, ROIs induced arrays of cellular protectant and defense genes and also cued the collapse of challenged cells^[10-12]. These findings prompted us to test whether the extracellular ROIs photoinduced by nano-TiO₂ semiconductors could facilitate the aforementioned functions and exhibit resistance to plants diseases.

Hitherto, there is still no literature that reports applications of nano-TiO₂ semiconductors to prevent from plant diseases *in vivo*. The aim of present study is to investigate the effects of nano-TiO₂ semiconductor sol on preventing from pathogen invasion and inhibit the development of plant diseases of bacterial angular spot (caused by *Pseudomonas syringae pv. lachrymans*) and downy mildew (caused by *Pseudoperonospora cubensis*) in agricultural production by forming antibacterial films on the surfaces of leaves. The results implicated that there would be a bright future to conquer pesticides pollution and improve food

*Corresponding author. Tel: +86-10-62139373;
E-mail: haixin_cui@hotmail.com.

safety by application of nano-TiO₂ semiconductors on plant epidemic diseases prevention.

2 Materials and methods

2.1 Materials

Cucumber seeds (*Cucumis sativus L.*) of *Zhongnong No. 118* (susceptible to bacterial angular spot) and *Jinlü No.2* (resistant to downy mildew) were purchased from the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences (CAAS). The seeds had been sowed in breeding plugs with one seed per cell after germinated at 28°C for 12 h in the dark. At cotyledon fully expanded stage, the seedlings (*Zhongnong No.118*) were planted in flowerpot (21 cm diameter, 18 cm deep), and used for inoculating experiments at 6th or 7th leaf-stage. The seedling growth medium, which was a 3 : 1 mixture of peat-based compost and vermiculite, was irrigated with Yamasaki culture solution and the seedlings were cultivated in greenhouse with temperature between 28°C and 18°C. For field experiments, the seedlings (*Jinlü No.2*) were planted in seedling-raising pot (7 cm diameter), and finally transplanted in field at 3rd or 4th leaf-stage, with 0.25 m plant-spacing and 0.65 m row-spacing.

Two kinds of bacterial pathogens, *Pseudomonas syringae pv. lachrymans* and *Xanthomonas vesicatoria*, provided by Plant Protection Institute of CAAS, were prepared separately to bacterial suspension with the concentration of approximate 10⁸ bacterium per milliliter, and then stored in refrigerator at 4°C.

Nano-TiO₂ semiconductor sol (nano-TiO₂ sol) was synthesized by sol-gel method in our laboratory. The main characters of nano-TiO₂ sol were given in Tab. 1.

TiO₂-coated glass slides (100 mm × 50 mm) used for antibacterial experiments were prepared by dipping in nano-TiO₂ sol once and then being fired at 160°C for 3 h in hot air oven.

Metalaxyl, a kind of fungicide used to prevent from downy mildew in field experiment, was bought from a local pesticide company.

2.2 Methods

2.2.1 Antibacterial ability assay

For evaluating the antibacterial properties of nano-TiO₂ sol, 0.3 mL aliquots of bacterium suspension was transferred to a TiO₂-coated glass slide and covered with a translucent plastic thin film, followed by be irradiated underneath 20 cm from the black light (40 wattages) for 12 h. Subsequently the treated glass slide was rinsed with 3 mL sterile water repeatedly. Then 0.1 mL of the rinsing solution was taken up to cultivate on nutrient agar (NA) culture medium. Colonies were counted after incubation at 27°C for 36 h. Normal glass slides were used for the control and each treatment had three repeats. Relative antibacterial rate of nano-TiO₂ sol was calculated according to the following formula.

Antibacterial rate (%) =

$$\frac{(\text{Clone numbers of control} - \text{Clone numbers of NTSS})}{\text{Clone numbers of control}} \times 100\%.$$

2.2.2 Plant inoculation experiment

The cucumber seedlings, *Zhongnong No.118* (20 flowerpots), at stage of 6th-7th leaves were sprayed with 0.35% nano-TiO₂ sol and with distilled water as the control. Each treatment was replicated three times, with the experiments arranged in randomized blocks. After nano-TiO₂ sol formed a continuous translucent film on the surfaces of leaves, bacterium suspensions of *P. s. pv. lachrymans* were inoculated by using a high pressure sprayer, then incubating the seedlings in a growing chamber with a controlled environment of (22 ± 1)°C in temperature and 80–100% in humidity for 48 h. Subsequently the seedlings were transferred into greenhouse and cultivated according to normal production processes. After 7 days of inoculation, cucumber leaves were secondly sprayed with the same sol to mend the splits of TiO₂-films which were caused by enlarged leaf areas.

About 18 days after inoculation, lesion numbers and areas on the leaves of individual plant were investigated by randomly selecting 15-seedlings from each block. The

Tab. 1 Main characters of nano-TiO₂ sol

Content of TiO ₂ particles (%)	Water content (%)	Grain pattern	Average grain sizes (nm)	pH value
1.4	98.6	anatase	30.6	7.0

Tab. 2 Relative antibacterial rate of nano-TiO₂ sol

Bacterial pathogens	Treatments	Survival (CFU)	Antibacterial rate(%)
<i>P. s. pv. lachrymans</i>	Control	1944 ±45.6A	–
	Nano-TiO ₂ sol	1.6 ±0.5B	99.9
<i>X. vesicatoria</i>	Control	1393.7±37A	–
	Nano-TiO ₂ sol	0B	100

Note: The values indicated with different letters are significantly different at $P=0.01$ by using t -test; Values are means±SE of 9 measurements.

severe degrees of bacterial diseases were assessed by using nine score to describe leaf infection and penetration (0: uninfected; 1: less than 5% lesion areas; 3: 6% ~ 10% lesion area; 5: 11% ~ 20% lesion area; 7: 21% ~ 50% lesion area; 9: more than 50% lesion area). The control efficiency of nano-TiO₂ sol to cucumber bacterial angular spots was evaluated by comparing leaves' disease incidences and disease indexes between blocks with different treatments. The methods of investigation and calculation referred to *GT/B 17980.110–2004* [13]. Besides, lesion areas of leaves between different treatments were compared by taking top 4th ~ 6th leaves as example.

2.2.3 Field experiment

At 7th or 8th leaf-stage, cucumber leaves (*Jinliu No.2*) in treated blocks were sprayed with 0.35% nano-TiO₂ sol using a manometric sprayer. Contrast experiments were conducted by spraying Metalaxyl and water respectively in different blocks. Each treatment set three blocks with square of 2.7 m × 2.0 m in random distribution, and each block with 40 plants. The spraying interval was 7 days. After being treated three times later, the investigation of plant fungal diseases was taken by randomly selecting 20 seedlings in each block. Methods of investigation and calculation were according simultaneously to *GB/T 17980.26–2000* [14] and the counterpart was at 1.2.2 portion.

2.2.4 Statistical analysis

Statistical analysis was done using SAS ver. 6.12 learn (SAS Systems, Cary, NC, USA). For evaluation the antibacterial properties of nano-TiO₂ sol, results were shown in the manner of $(\bar{x} \pm SE)$. Significance of differences be-

tween control and nano-TiO₂ sol treated samples was determined by analysis of variance followed by t -test at the 0.01 level of significance. For inoculating experiment and field experiment, results were analyzed statistically by analysis of variance (ANOVA). When analyzing variance treatment effect ($P=0.05$), the least standard deviation (LSD) test was applied to make a comparison between means at the 0.05 level of significance.

3 Results

3.1 Loss of viability under nano-TiO₂ photocatalytic reaction

It was observed that nano-TiO₂ sol synthesized in experiments had a perfect adhesive and film forming ability and it could form continuous and stable films on the glass surfaces. The viabilities of TiO₂-treated pathogenic bacteria (*P. s. pv. lachrymans* and *X. vesicatoria*) were determined by colony counting after 36 hours of incubation. It showed clearly in Tab. 2 that more than 99% of bacterial cells (approximately 10⁸ cfu·mL⁻¹) lost their viabilities after being illuminated for 12 h on the surface of TiO₂-coated glass. The relative antibacterial ability of nano-TiO₂ sol to *P. s. pv. lachrymans* and *X. vesicatoria* was 99.9% and 100% respectively. It was confirmed in the experiment that nano-TiO₂ semiconductors had a powerful antibacterial ability without significant selectivity concerning to tested pathogenic cells.

3.2 Effects on preventing from plant bacterial diseases

The effects of nano-TiO₂ sol on preventing from plant bacterial diseases was examined through spraying TiO₂ sol before inoculating pathogenic bacteria on the leaves of

Tab. 3 Investigation of control efficiency of nano-TiO₂ sol to *P. s. pv. lachrymans* (unit: %)

Experimental treatments	Lesion areas			Disease incidences	Disease indexes	Control efficiency
	4 th leaves	5 th leaves	6 th leaves			
Control	13.3 a	18.0 a	12.07 a	68.34 a	14.5 a	–
Nano-TiO ₂ sol	3.87 b	6.47 b	5.6 b	42.68 b	6.29 b	56.62

Note: The value indicated with different letters were significantly different at $P=0.05$ by using LSD. The same as below.

Zhongnong No.118. Fully expanded leaves, after infected with pathogen of *P. s. pv. lachrymans*, appeared water-soaked lesions and progressive development of disease symptoms, even the perforating lesions. Results in Tab. 3 showed that lesion areas of 4th to 6th leaves, disease incidences and disease indexes in TiO₂-treated blocks were significantly less than that of contrast blocks. The control efficiency of nano-TiO₂ sol to *P. s. pv. lachrymans* is 56.62%. It was also observed that the procedure of localized cell death was more quickened on TiO₂-treated leaves than untreated ones. By this means, nano-TiO₂ films destroyed the invasive bacterial cells and prevented the development and pervasion of *P. s. pv. lachrymans* at the surrounding healthy tissues.

3.3 Effects on preventing from plant fungal diseases

For field experiment, cucumber plants (*Jinlü No.2*) had been transplanted in fields on Aug. 27, 2005, and started to spray TiO₂ sol on Sep. 15, 2005. Before the spraying treatments, no diseased phenomenon was observed on cucumber leaves in fields. Cucumber plants treated three times later, had been investigated the downy mildew on Oct. 9, 2005. It was showed in Tab. 4 that leaf disease incidences, indexes and lesion areas and numbers on 7th–9th leaves in water-treated blocks were significantly higher than TiO₂ and Metalaxyl treated blocks. The control efficiency of nano-TiO₂ sol and Metalaxyl to *P. s. pv. lachry-*

mans were 80.09% and 87.52% respectively. ANOVA showed no significant differences between blocks treated with TiO₂ sol and Metalaxyl. Furthermore, it was observed that the disease spreading speed from lower leaves to top-pers had also been slowed down in TiO₂-treated blocks. It was confirmed that nano-TiO₂ sol had the same efficiency with Metalaxyl to prevent the development of plant fungal diseases.

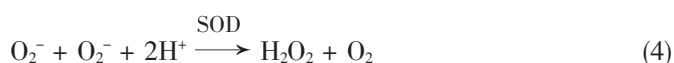
4 Discussion

The experimental evidences show that nano-TiO₂ semiconductors have intensive antibacterial ability without significant selectivity concerning to tested pathogenic cells. This results from the properties of TiO₂ semiconductor, which can be excited under irradiating and produce an electron and a hole on its CB and VB respectively (1). Meanwhile, a single semiconductor can be thought as a short-circuited photoelectrochemical cell providing both oxidizing and reducing sites for the reaction [7]. At the oxidizing site, a water molecule is oxidized to hydroxyl radical (OH·) (2). At the reducing site, it is speculated that the photogenerated electrons mainly react with molecular oxygen to form O₂⁻ under aerial condition (3) [7]. The OH·, more reactive among ROIs, can react with various chemical species and drive chemical reactions. It is presumed that OH· can directly photodegrade bacterial cell wall and

Tab. 4 Investigation of control efficiency of nano-TiO₂ sol to *P. cubensis* (unit: %)

Experimental treatments	Lesion areas			Disease incidences	Disease indexes	Control efficiency
	7 th leaves	8 th leaves	9 th leaves			
Water	18.77 a	26.43 a	33.5 a	76.19 a	39.17 a	–
Nano-TiO ₂ sol	4.47 b	6.37 b	8.5 b	26.78 b	7.8 b	80.09 a
Metalaxyl	2.33 b	4.27 b	5.37 b	18.24 b	4.89 b	87.52 a

cytoplasmic membrane^[5-8]. Once the integrity of the cell envelope becomes compromised, intracellular components begin to leak from the cell. Therefore, pathogenic cells will soon lose viability when attached to the irradiating TiO₂-film. In general, photocatalytic produced O₂⁻ is less reactive and has a short lifetime^[15-18]. It is assumed that O₂⁻ can be catalyzed to become more reactive hydrogen peroxide (H₂O₂) with the aid of superoxide dismutase (SOD) enzyme existing in the plasma membranes of plant cells (4)^[7,19].



Furthermore, it has been interestingly found that O₂⁻^[10, 15-18, 20] is the first product of the oxidative burst in plant responding to pathogen attack. Although it is a poor candidate for the cell executioner, because it has a short half-life, being rapidly converted to H₂O₂, and it does not readily diffuse, nevertheless as a trigger, O₂⁻ may ignite the signal transduction to establish plant immunity. In this case, cell permeant radicals, e.g., H₂O₂, accumulating at the site of oxidative burst^[11], diffusing between and through cells and rapidly metabolizing, plays a central and integrative role in orchestrating the expression of hypersensitive response^[17]: (a) as the substrate driving the cross-linking of cell wall structural proteins to slow microbial ingress and to trap pathogens in cells destined to undergo hypersensitive cell death^[21], (b) as a local threshold trigger of programmed death in challenged cells, and (c) as a diffusible signal for the induction in adjacent cells of genes encoding cellular protectants. Accumulation of H₂O₂ to a threshold level sufficient to trigger local cell death requires a rapid and massive activation of the oxidative burst machinery^[10, 11, 22]. Thus, induction of massive and prolonged ROIs by photoexcited TiO₂-films on the surfaces of leaves, which closely resemble the oxidative burst produced by cells undergoing pathogen attack, may induce the prolonged hypersensitive disease resistance in plants. Whether these functional similarities reflect similar mechanisms is not understood well. And it is still not clearly whether the nano-TiO₂ sol sprayed on leaves prevents the plant diseases directly (by killing cells^[7, 9, 11]), indirectly (by signal-

ing further cellular responses), or both. Further studies may therefore give insights into the resistant mechanism of agricultural plants by supplying with the nano-TiO₂ semiconductor sol.

5 Conclusions

The following conclusions were confirmed in the researching experiments:

- (1) The nano-TiO₂ semiconductor sol can form a successive, adhesive and transparent film on surfaces of leaves after spraying treatment;
- (2) The film of nano-TiO₂ semiconductor has powerfully antibacterial effects to pathogens;
- (3) No significant difference exists in selectivity of TiO₂ semiconductor to various tested pathogens;
- (4) The TiO₂ semiconductor sol can delay and control the development of cucumber diseases of bacterial angular spot and downy mildew.

These results indicate that nano-TiO₂ semiconductor sol may be developed as environment-friendly fungicide in preventing and controlling the development of plant bacterial / fungal diseases.

Acknowledgment

This work was supported by national 863 progra(Grant No. 2006AA10A203).

References

- [1] Fujishima A., Honda K.. Electrochemical photolysis of water at a semiconductor electrode [J]. *Nature*, 1972, 238: 37-38.
- [2] Obee T.N., Brown R.T.. TiO₂ photocatalysis for indoor air applications: effects of humidity and trace contaminant levels on the oxidation rates of formaldehyde, toluene, and 1, 3-butadiene. *Environmental Science and Technology* [J]. 1995, 29 (5): 1223-1231.
- [3] Bahnamann D.. Mechanism study of water detoxification in illuminated TiO₂ suspensions [J]. *Solar Energy Materials*, 1991, 24 (3): 564-583.
- [4] Fujihira M., Satoh Y., Osa T.. Heterogeneous photocatalytic oxidation of aromatic compounds on TiO₂ [J]. *Nature*, 1981, 293 (2): 206-208.
- [5] Matsunaga T., Tomoda R., Nakajima T., *et al.* Photochemical sterilization of microbial cells by semiconductor powders [J]. *FEMS Microbiol. Lett.*, 1985, 29: 211-214.
- [6] Matsunaga T., Tomoda R., Nakajima T., *et al.* Continuous-sterilization system that uses photosemiconductor powders [J]. *Applied and Environmental Microbiology*, 1988, 54(6): 1330-1333.

- [7] CAI R.X., Hashimoto K., Kubota Y., Fujishima A.. Increment of photocatalytic killing of cancer cell using TiO₂ with the aid of superoxide dismutase [J]. *Chemistry Letters*, 1992, 3(3): 427–430.
- [8] HUANG N.P., HUANG D., XU M.H., *et al.* The study of the photokilling effect and mechanism of ultrafine TiO₂ particles on U937 cells [J]. *Biochemistry and Biophysics*, 1997, 24(5): 470–473.
- [9] Sunada K., Kikuchi Y., Hashimoto K., *et al.* Bactericidal and detoxification effects of TiO₂ thin film photocatalysts [J]. *Environmental Science and Technology*, 1998, 32(5): 726–728.
- [10] Lamb C., Dixon R.A.. The oxidative burst in plant disease resistance [J]. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 1997, 48: 251–275.
- [11] Levine A., Tenhaken R., Dixon R.A., *et al.* H₂O₂ from the oxidative burst orchestrates the plant hypersensitive disease resistance response [J]. *Cell*, 1994, 79: 583–593.
- [12] Jabs T., Dietrich R. A., Dangl J. L.. Initiation of runaway cell death in an Arabidopsis mutant by extracellular superoxide [J]. *Science*, 1996, 273: 1853–1856.
- [13] *GB/T 17980. 110–2004*, Pesticide–guidelines for the field efficacy trials (II)–part 110: fungicides against bacterial angular leaf spot of cucumber [S].
- [14] *GB/T 17980. 26–2000*, Pesticide–guidelines for the field efficacy trials (I)–fungicides against downy mildew of cucumber [S].
- [15] Dangl J.. Innate immunity: Plants just say NO to pathogens [J]. *Nature*, 1998, 394: 525–527.
- [16] Alvarez M.E., Pennell R.I., Meijer P.J., *et al.* Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity [J]. *Cell*, 1998, 92: 773–784.
- [17] Tenhaken R., Levine A., Brisson L., *et al.* Function of the oxidative burst in hypersensitive disease resistance [J]. *Pro. Natl. Acad. USA*, 1995, 92: 4158–4163.
- [18] CHEN Z., Silva H., Klessig D.F.. Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid [J]. *Science*, 1993, 262: 1883–1886.
- [19] Cakmak I., Marschner H.. Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase in bean leaves [J]. *Plant Physiol*, 1992, 98: 1222–1227.
- [20] Sutherland M.W.. The generation of oxygen radicals during host plant response to infection [J]. *Physiological and Molecular Plant Pathology*, 1991, 39: 79–93.
- [21] Bradley D.J., Kjellbom P., Lamb C.J.. Elicitor– and wound–induced oxidative cross–linked of a plant cell wall praline–rich protein: a novel rapid defense response [J]. *Cell*, 1992, 70: 21–30.
- [22] Beatwick C.S., Brown I.R., Bennett M.H.R., *et al.* Localization of hydrogen peroxide accumulation during the hypersensitive reaction of lettuce cells to *Pseudomonas syringae* pv. *phaseolicola* [J]. *Plant Cell*, 1997, 9: 209–221.