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Oxidative photo-catalyzed degradation of a new biological fungicide, phenazine-1-carboxylic acid

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ABSTRACT

Phenazine-1-carboxylic acid (PCA) is a secondary metabolite produced primarily by *Pseudomonas* spp. and has been used as a potential fungicide towards many soil-borne fungal phytopathogens due to its broad-spectrum antibiotic activity. The present study aimed to investigate the photo stability of PCA to different types of light sources and degradation behavior in different environments, including at pH values and oxidants existing in the solution. Results showed that PCA was found to be very stable in a dark environment. However, UV radiation and visible light could induce its degradation and the photochemical reaction rate increased in the different solutions with the following order: methanol > ethyl acetate > acetone> phosphate buffer solution (pH 6.8). Analysis of pH profile showed that PCA becomes quite unstable towards decreasing the pH value of the environment. During the photo degradation of PCA, singlet oxygen was quenched by the addition of sodium azide and no measurable super oxide anion was produced, which suggests that the photo degradation of PCA was photo sensitized oxidation by the type II pathway. Moreover, oxygen and hydrogen peroxide could also enhance the photo degradation of PCA in an aqueous solution. The data obtained from this study would be beneficial for the future application of PCA in agricultural production processes.

Keywords: Phenazine-1-carboxylic acid; Photo degradation; Singlet oxygen; Photo sensitized oxidation

1. Introduction

Phenazines are heterocyclic, nitrogen-containing brightly colored pigments with multiple absorption peaks in the UV and visible ranges that vary according to the nature and position of substituents on the ring [1,2]. To date, more than 6000 phenazine derivatives have been identified and reported [3]. Among these compounds, up to 50 are of natural origin and synthesized by a wide variety of soil and marine habitats, such as *Pseudomonas* and *Streptomyces* [4]. The noteworthy biological properties of these natural products include antibiotic, antitumor, antimalarial and anti parasitic activities [1,5–9]. Also, natural phenazine products display great promise for use as electron donors and acceptors, constituents of microbial fuel cells (MFCs), environmental sensors and biosensors, and vital components of antitumor compounds development [1].

Phenazine-1-carboxylic acid (PCA) is a well-characterized key intermediate in the biosynthesis pathway of phenazines and can further be transformed to other value-added phenazine derivatives such as pyocyanin (PYO), 1-hydroxy phenazine, 2-hydroxy phenazine-1-carboxylic acid (2-OH PCA), and phenazine-1-carboxamide (PCN) by different terminal-modifying enzymes [10]. Most of them possess broad-spectrum antibiotic activity toward soil-

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borne fungal phyto pathogens and could act as elicitors of induced systemic resistance (ISR) in the plant rhizo sphere [6,11]. Tomashow and Weller, [12] demonstrated that PCA was a major determinant in the ability of *Pseudomonas fluorescens* 2–79 to suppress take-all disease, an important root and crown rot of wheat caused by *Gaeumannomyces graminis* tritici. Considering the relative nontoxicity to animals, PCA has been registered as a herbicide and algaecide by U.S. patent 3367765 issued in 1968 [13]. In 2011, PCA has been commercialized as "Shenqinmycin" due to its high efficiency and good environmental compatibility and has gained a Pesticide Registration Certification issued by the Ministry of Agriculture of China (PD20110314, PD20110315).

The stability of PCA is an important issue for its agricultural application and environmental behavior. The antibiotic activity of PCA against soil-borne phyto pathogens in the crop field was found to be less than that in laboratory studies, possibly because of degradation in the crop field testing. For instance, Chen et al. [14] reported a biological degradation of PCA by *Sphingomonas* sp DP58 which was screened from the plant root-soil in a Shanghai suburb field. Zhao et al. [15] also recorded the PCA degradation when it was used to control the fungus *Phytophthora*. The half-life of PCA degradation in the soil and on the leaves of capsicum was 8.0~8.7 and 5.4~6.6 d, respectively. It indicates that PCA is degraded more quickly on the plant than in the soil and the sunlight might have an important effect on PCA efficiency. However, there is no knowledge about the photostability of PCA; only a few reports regarding photodegradation of PYO, neutral red and Janus green were found in the literature [16]. PYO is another phenazine secondary metabolite produced by *Pseudomonas aeruginosa*, which is rapidly and non-reversibly photo inactivated with first-order kinetics to produce colorless photo products [17,18]. In order to improve the effectiveness of PCA in crop fields, it is indispensable to study the stability of PCA to light under the natural conditions. Therefore, the present work aimed to acquire information about the photo stability of PCA to different types of light sources and degradation behavior in different environments.

2. Materials and methods

2.1. Chemicals and reagents

PCA (>95% purity) used in this study was prepared by our laboratory (Laboratory of Microbial Resources and Metabolic Engineering) [19], whereas the methanol (HPLC grade) was provided by the Lingfeng Chemical Reagent Co. Ltd., China. All other chemicals were of analytical grade and mainly purchased from the Sinopharm Chemical Reagent Co. Ltd. China. Ultra-pure water (18.2 Ω cm) used throughout the experiment was produced by a Milli-Q water system (Shanghai Laikie Instrument Co., Ltd, China).

2.2. Light sources and experimental setup

A fluorescent lamp (58 W color temperature 6500 k Philips Electronics & Lighting, Inc., China) was used as the source of visible light with a light intensity of about 40390-710 LUX. The photon flux density of visible light was measured with a TES 1339 light Meter (TES Electrical Electronic Corp. China). A UV lamp (25 W Philips Electronics & Lighting, Inc. China) served as the source of UV radiation. Light intensities were adjusted by the distance between the samples and light sources. The samples exposed to sunlight were placed on the roof of our laboratory building, when the temperature varied from 17°C to 26°C with an average temperature of 22°C. During the experimental period, there were eleven sunny days and one rainy day. The photochemical reactions irradiated by UV or visible light were carried out at a temperature of 28°C in the room. The photon flux density of visible light was 727.0 ± 12.8 μ mol/(m^2·s). The power of UV radiation was 25 W and the distance between samples and axis of the UV lamp was 5 cm.

2.3. Photochemical reactions of PCA

Due to the poor solubility of PCA in pure water, a phosphate buffer solution (PBS 0.2 M, pH 6.8) was used to dissolve PCA to a moderate concentration. The pH value of the buffer solution was adjusted with HCl or NaOH and measured with a PHS-25 Analog pH meter (Shanghai Precision Scientific Instrument Co., Ltd, China). All of the PCA solutions were kept stationary for 12 h in the dark before exposure to the light. All of the reactions were carried out in cylindrical glass reactors (25 mL, Shanghai Heqi Glass Instrument Co., Ltd., China) with a volume of 10 mL under irradiation of different lights. At the same time, reactors for experimental control were packed with black paper and kept in the dark under the identical conditions. Samples were collected regularly at certain time intervals and analyzed by high-performance liquid chromatography (HPLC). All the experiments were performed in triplicate.

2.4. Analytical procedure

The concentration of PCA was measured by HPLC (Smartline 1000 Knauer, Germany) with a UV-Vis diode detector (Smartline 2600 Knauer, Germany) and an Eclipse XDB-C18 column (46 × 250 mm, 5 μm particles of packing material Agilent, US). The mobile phase containing methanol + 0.1% acetic acid solution (70 + 30 by volume) was used at a flow rate of 1.0 mL/min. The eluent was monitored under the spectral peak maxima (254 nm and 268 nm), which are characteristic of PCA in the designated solvent system. The retention time of PCA was 9.5 min under these conditions.

3. Results and discussion

3.1. Photo stability of PCA in different solvents

Solvent, as the main component of the pesticide product, is an important factor which affects the pesticide photo stability. Therefore, the photo stability of PCA in different solvents such as methanol, ethyl acetate, acetone, and PBS (pH 6.8) was investigated. These solutions are the common solvents used as the ingredients of some conventional pesticide formulations. The initial concentration of PCA used was 111.4 ± 6.9 mg/L. One group of samples was placed under fluorescent lamp illumination, whereas another

group of samples was placed in the dark as controls under the identical conditions. The proportion of residual PCA under visible light irradiation is shown in Fig. 1. It was found that concentrations of PCA gradually decreased in all the tested solvents when exposed to visible light. However, the negligible change was observed when PCA was placed in a dark environment. More than 98% of the remaining PCA in the control solutions after 15 d storage indicates its instability towards the visible light and the degradation was light-dependent.

The photochemical reaction rate increased in the different solutions with the following order: methanol > ethyl acetate > acetone> PBS (pH 6.8). After 1 d, the concentration of PCA in the methanol decreased by 90%. In the PBS (pH 6.8), PCA showed the best photo stability among the four different solvents. After 15 d irradiation by visible light, the level of PCA decreased by only 24.1%. For this reason, the PBS was chosen as the solvent in the subsequent experiments.

When the photochemical reaction solutions were analyzed by HPLC, new peaks were detected in the four solvents. The photo products were more complex in organic solvents than that in PBS and were quite different from each other. It seems that the photochemical reaction rate, pathways, and photo products of PCA rely profoundly on the solvents used, just like the photo degradation of the organo phosphorus herbicide HW-02 [20]. At the end of irradiation, the PCA solution becomes colorless and no PCA derived chemicals could be apparently detected by the HPLC/UV diode array detector. The result revealed that the photo degradation of PCA was an irreversible process and the phenazine chromophore was disrupted in the reaction.

3.2. Effect of different light sources on PCA photo degradation

In the pesticide industry, many products are not stable to light. The light of different wavelength and light intensity could exert different influences on the stability of pesticides. PYO was intensely photo degraded with 371 and 420 nm light and there was little or no effect when exposed to 550, 660 and 740 nm light [17]. Here, the influence of different light sources including sunlight, UV radiation, and visible light was investigated in PBS (pH 6.8) and results are shown in Fig. 2. Evidently, PCA was degraded quickly when exposed to all of three kinds of light (Fig. 2). The UV radiation and visible light can trigger the photochemical reaction of PCA in aqueous solution. Under the experimental conditions, UV radiation had a stronger effect on the photo stability of PCA. In PCA spectroscopy, there are three main absorption peaks at 215, 249, and 371 nm. Among these peaks, PCA has the largest absorbance coefficient at 215 nm [21]. Since the energy to break chemical bonds in pesticide molecules usually ranges from 70 to 120 kcal/mol corresponding to light at wavelengths of 250–400 nm, UV radiation has a stronger influence on PCA stability [16]. Consequently, PCA could absorb more energy with UV radiation and short wavelength would enhance its photo degradation.

3.3. Influence of pH on PCA photo degradation

The pH is another important factor in the stability of pesticide. Though no significant differences was reported in photo degradation efficiency at different pH environments for many conventional pesticides [22,23], pyoluteorin (another biological pesticide, Plt) was found to be photo degraded with different rates in aqueous solutions of varying pH ranged from 5.8–7.8. The half-life of Plt was 34.8 d at pH 5.8, while it decreased to about 24 days when the pH was changed to 7.8 [24]. Therefore, the photo stability of PCA was studied in solutions with the pH values varying from 5.0 to 8.0. The range of pH values chosen is similar to that of typical soil pH values in China.

The control samples were placed in the dark with the same PCA concentrations using the identical pH value. After 60 d, the percentages of the residual PCA in these control samples were above 95% in all the experiments and no degradation products could be detected by HPLC. On the other hand, the samples exposed to visible light were degraded with different reaction rates (Fig. 3). The PCA was observed to be quite unstable in the environment at lower pH values,

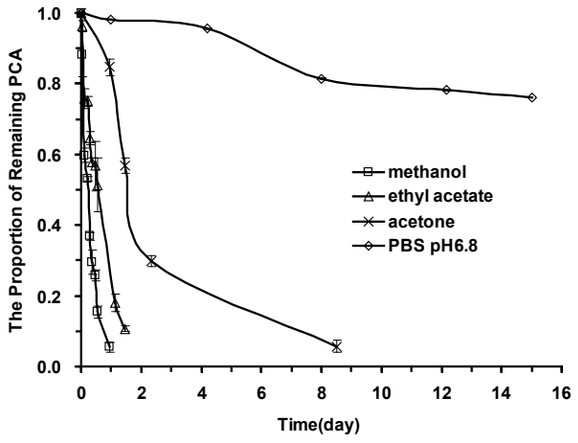

Fig. 1. The photo stability of PCA in different solvents irradiated by visible light (□ methanol, Δ ethyl acetate, x acetone, ◊ PBS pH 6.8).

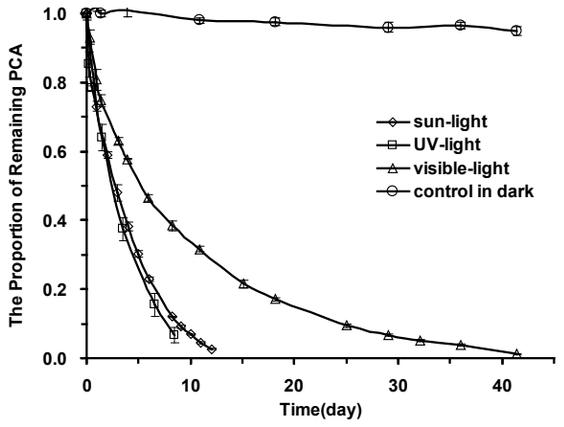

Fig. 2. The photodegradation of PCA exposed to different light sources (□ UV radiation, Δ visible light, ◊ sunlight, ○ control in dark).

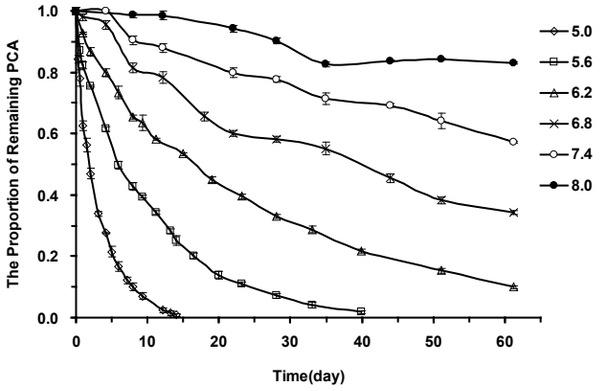

Fig. 3. The photo degradation of PCA in the solutions with different pH values (◊ 5.0, □ 5.6, Δ 6.2, x 6.8, ○ 7.4, • 8.0).

whereas it showed stability with an increase of the environment pH value. It indicated that the hydrogen ion concentration has a critical impact on the stability of PCA when it exposed to visible light. After irradiation under visible light for two months, the percentages of the remaining PCA were still above 50% in the solutions with pH 7.4 and 8.0.

The reason for photo degradation influenced by the pH value was possibly due to the charge status of PCA. The protonated PCA may be more photolabile than its unprotonated molecule like the degradation of N-nitroso dimethylamine [25]. The pH value of the environment not only affects the photo stability of PCA but also affects its bioactivity. It was reported that the inhibition of *Gaeumannomyces graminis tritici* by purified PCA was greater at pH 6.0 than that at higher pH values [26].

3.4. Quenching of ROS

Generally, photochemical reactions are categorized into "direct" and "indirect" photolysis. Among these reactions, photo sensitized oxidation is one of the most important reaction types of pesticide photo degradation. It can be explained by type I and/or type II mechanistic pathways depending on the photo sensitizer and environmental conditions. The type I reaction results in hydrogen atom or electron transfer yielding radicals or radical ions and type II pathway leads mainly to singlet oxygen by energy transfer or forming super oxide anion. Therefore, the effect of sodium azide (a physical singlet oxygen quencher) and super oxide dismutase (SOD, an enzyme capable of specifically inactivating super oxide anion) [27] on the photo degradation of PCA was examined.

Fig. 4 portrays that the photo degradation of PCA markedly decreased when sodium azide was added to the system. However, SOD shows no influence on the photochemical reaction of PCA. Compared with control experiments and 5 mg sodium azide in the photochemical reaction system with the volume of 10 mL, the addition of 10 mg and 20 mg sodium azide evidently decreased PCA photo degradation. It implies that singlet oxygen was produced when PCA was exposed to visible light in PBS. The photochemical reaction of PCA was photo sensitized oxidation and its photo degradation was a process with the type II pathway. This result

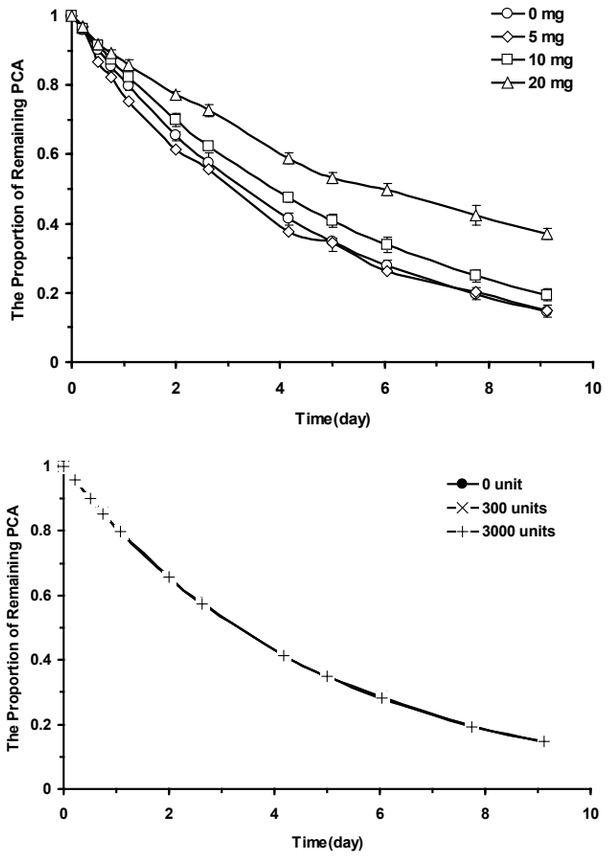

Fig. 4. The effect of ROS quencher on the photo degradation of PCA in 10 mL PBS (A), the addition of NaN_3 ○ 0 mg, ◊ 5 mg, □ 10 mg, Δ 20 mg; (B), the addition of SOD (• 0 unit, x 300 units, + 3000 units).

was in agreement with the photo sensitized oxidation of PYO, which is oxidized largely via singlet oxygen.

In the type II pathway, photo sensitized oxidation produces not only singlet oxygen, but also super oxide anion. In this experiment, the addition of SOD with 300 and 3000 units did not show any change in photo degradation of PCA. These results suggest that super oxide anion was not involved in the photo sensitized oxidation of PCA. However, other phenazine dyes show different photo degradation mechanisms. Neutral red which exhibited high photo activity against *Streptococcus aureus* produced no measurable singlet oxygen during in vitro testing and suggests a type I photo sensitization pathway. Similarly, Janus green has been shown to be a photo sensitizer in mammalian cells and presumably acts via a type I pathway [16]. Hence, it would be better to produce a formulation with some of the photo sensitized inhibitors such as sodium azide to make the PCA last longer in the field.

3.5. Effect of oxygen on PCA photo degradation

It was reported that reduced phenazine derivatives could react with O_2 and the reactivity decreased in the order: PYO > 1-hydroxy phenazine > PCA [18]. In addition,

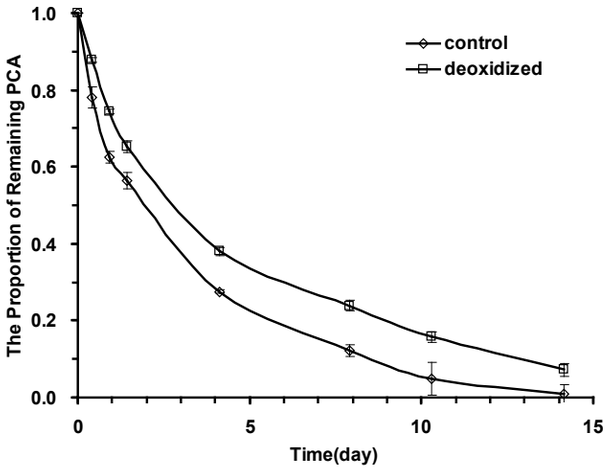

Fig. 5. The effect of oxygen on the photo degradation of PCA (□ the deoxidized sample flasks were purged with nitrogen gas for 10 min firstly before the irradiation by light. ◊ Control experiment: the degradation solution of PCA was not purged by nitrogen gas).

since singlet oxygen was proposed as the oxidant of photo degradation, the effect of oxygen concentration in PBS on PCA photo stability was studied by the injection of nitrogen to the PCA solution. The samples without degassing were used as a control. Fig. 5 portrays that PCA concentration was decreased to a lesser degree than that in the control experiments because of oxygen removal from the solution. It indicated that oxygen in the solution was an oxidative component and it would accelerate the photo degradation of PCA during the storage and usage.

3.6. Addition of H_2O_2

Phenazines are a class of secondary metabolite molecules produced by a variety of microorganisms [1,7–8,28–30]. Beyond merely serving as antibiotics, some studies show that phenazines play important physiological roles based on their redox-active properties. The phenazines could be reduced by NADH or oxidized by H_2O_2 in the cell [1]. Reszka et al. [31] reported that PYO undergoes oxidative inactivation by hydrogen peroxide and microperoxidase 11 or hemin, as evidenced by loss of the pigment's characteristic absorption spectrum. In order to investigate the influence of oxidant on PCA photo stability, H_2O_2 was added to a PCA solution with an initial concentration of 97.8 mg/L and exposed to the visible light at 28°C. The molar ratios of H_2O_2/PCA varied from 0.25:1 to 4:1 and the results are shown in Fig. 6.

As a control, the samples with the same molar ratios of H_2O_2/ PCA were placed in the dark. Nine days later, the percentages of remaining PCA were respectively 99.1%, 95%, 88.7%, 84.3%, 76.3% and 63.5% corresponding to the molar ratios of H_2O_2 to PCA by 0:1, 0.25:1, 0.5:1, 1:1, 2:1 and 4:1 separately. PCA was found to be stable without H_2O_2 in the dark. With the increased concentration of H_2O_2 in the solution, PCA disappeared quickly in the light samples. It was confirmed that PCA is a kind of redox-active antibiotic and the stability was affected by the oxidant in the environment. Based on these results, it appears that the oxidation of PCA can occur with H_2O_2 in a reaction independent of photo degradation but considerably slower than the photo degradation process.

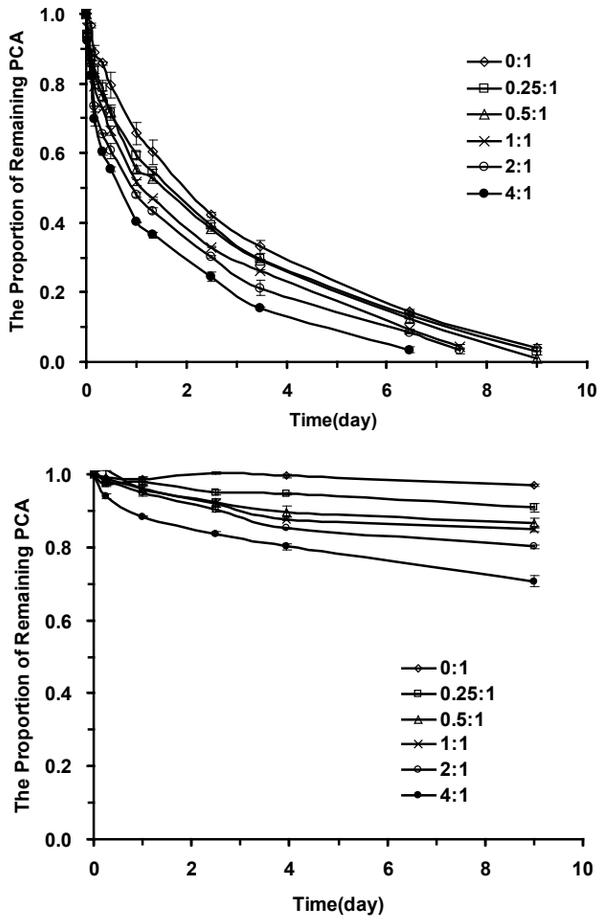

Fig. 6. Effect of H_2O_2 on the photo degradation of PCA (A), control in dark (B) irradiation by visible light (◊ 0:1, □ 0.25:1, Δ 0.5:1, x 1:1, ○ 2:1, • 4:1).

Fig. 6 showed that when the mixed samples were exposed to visible light, PCA was degraded faster than that in the dark. The visible light could enhance the oxidative degradation of PCA obviously. With the increase of molar ratios of H_2O_2/PCA, PCA became more unstable. From the HPLC graph of oxidative degradation and photo degradation, the retention time of new peaks was as same regardless of whether there was light irradiation. It seems that oxidative reaction by H_2O_2 and photo degradation of PCA possibly shared the same mechanism.

4. Conclusions

The present study investigated the photo stability of PCA to different types of light sources and degradation behavior in different conditions. Notably, the PCA was observed to be very stable in the dark environment, whereas UV radiation and visible light induced its degradation. The photochem-

ical reaction rate was increased as methanol > ethyl acetate > acetone > PBS (pH 6.8). At the pH values ranging from 5.0 to 8.0, PCA becomes quite unstable when the pH value of the environment decreases. During PCA degradation, singlet oxygen was quenched by the addition of sodium azide and no measurable super oxide anion was produced. Moreover, oxygen and H_2O_2 also caused the photo degradation of PCA in an aqueous solution. In conclusion, the findings of this study would be useful for the future application of PCA in agricultural production processes.

Acknowledgements

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Competing interests

The authors declare that they have no competing interests.

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