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A novel anti-counterfeiting method: Application and decomposition of RB for broad bean seeds (*Vicia faba* L.)

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ABSTRACT

Combining with seed coating method, rhodamine B (RB), a kind of fluorescent compounds had been used in seed labeling as an anti-counterfeiting technology. Effects of broad bean seed coating with RB on physiological parameters and fluorescence performance in broad bean seedlings were performed. The prolonged fluorescence in vascular bundles of seedlings treated with RB could be used as a marker of seed anti-counterfeiting. The experiment showed that RB had no negative effect on seed germination, seedling growth, seedling protective enzyme activities, and the contents of malondialdehyde (MDA) and chlorophyll, after coating seeds with ratio 1 kg RB to 10–30 kg seeds. In addition, the RB adsorption by soil was accorded with the isothermal adsorption equations of Langmuir, which exhibited the linear correlation as 0.9959, the maximum adsorption as 1.716 mg/g, and the adsorption equilibrium constant as 0.042 L/mg. The soil adsorption of RB could be effectively degraded by UV/Fenton. Present research suggested that coating broad bean seeds with appropriate concentration RB were safe to seed germination and this technology was suitable for anti-counterfeiting application.

1. Introduction

Broad bean (*Vicia faba* L.) is one of the most important legume crops in the market of the world, which increased the demand of seeds quality. To reduce the damage caused by fake seeds (Guan et al., 2011), many anti-counterfeiting methods have been developed. Traditional external packaging methods caused many drawbacks, such as reproducibility, short period, which restricted technology application. (Zhang et al., 2007a; Cai, 2009; Wang, 2009).

Fluorescent dyes had been used in plant research. Hapner and Hapner (1978) reported the detection of hem agglutinin in root tips by rhodamine fluorescence performance, and the observation of pollen propagation by fluorescent dyes during the 1970s (Waser and Price, 1982). The rhodamine 123 was used to test mitochondria of cabbage apical in the process of dehydration (Wu, 1987). The fluorescent dyes also showed that water entered the wheat germ firstly, then the ventral groove and finally the end of hairs along the nucellar layer and aleuronic layer (Shi, 2001). Moreover, HPTS (8-hydroxy, 3, 6-three acid pyrene), one fluorescent indicator, was successfully used to trace the route of nutrients into the seed coat (Joostt et al., 2003).

The fluorescent dye applying in seeds anti-counterfeit was firstly reported by Guan et al. (2011). With the seed coating technology, fluorescent dyes could be combined with seed coating agent, which provide an effective anti-counterfeiting method according to whether the fluorescence from seed or seedling existed or not. This fluorescence was not observed by naked eye. However, whether fluorescent dye will cause soil pollution and it will be decomposed in soil is lack of report.

The purposes of this paper were (a) to investigate the application of RB in seed anti-counterfeit of broad bean by observation the phytotoxicity effects of RB on seed germination and the dynamic characteristics of fluorescent in seedling; (b) to realize how RB was decomposed in soil environment.

2. Materials and methods

2.1. Plant materials

Broad bean seeds of cv. Japan wampee, from Lvwawa Seed Company, Hangzhou, China, were used as experimental materials. Base

Abbreviations: POD, peroxidase; CAT, catalase; SOD, superoxide distamuse; MDA, malondialdehyde; RB, rhodamine B.

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seed coating agent was obtained from Seed Science Center of Zhejiang University, China. Fluorescent dye, rhodamine B (RB), was purchased from Aladdin Company, Hangzhou, China. Seed coating agent were obtained from base coating agent added with RB (500 mg/L). Field soil, from farm of Zhejiang University, Hangzhou, China.

2.2. Seed labeling

Broad bean seeds were coated with seed coating agents containing RB, in which the mass ratio of coating agent to broad bean seeds were 1:10, 1:20, 1:30, respectively. Meanwhile, broad bean seeds coated with base seed coating agent (without adding RB) were used as controls, including CK1 (mass ratio of coating agent to seeds was 1:10), CK2 (mass ratio of coating agent to seeds was 1:20), CK3 (mass ratio of coating agent to seeds was 1:30). Considering the effect of base seed coating agent, the naked broad bean seeds were also used as a control, expressed by CK.

2.3. Seed germination and seedling growth

After labeling, 50 seeds were placed in a germination box with 300 g field soil. Each of the four replicates comprised of 50 seeds. Then germination boxes were incubated in a growth chamber under alternative cycle of 12 h light and 12 h darkness at 20 °C for 14 days and the germinated seeds were recorded daily. Germination energy and percentage was calculated on the 4th and 14th day, respectively (ISTA, 2004). After germination for 14 days, root length and shoot height were manually measured on twenty randomly selected seedlings with a ruler. Seedling dry weight was determined after drying at 80 °C for 24 h (Zhang et al., 2007b). The germination index ($GI = \Sigma(Gt/Tt)$) and vigor index ($VI = GI \times$ seedling weight) were calculated according to Hu et al. (2005), where Gt is the number of germinated seeds on days, Tt is time corresponding to Gt in days, and Σ is the sum.

2.4. Detection of seedling protective enzymes, MDA and chlorophyll content

The activities of peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX), superoxide distamuse (SOD) and the content of malondialdehyde (MDA) were measured using 8-day-old seedlings according to references (Song et al., 2004; Hu et al., 2005). The chlorophyll content was determined according to (Zhang et al., 2007a). The fresh seedlings were chopped fine and weighted 0.2 g by an analytical balance, then homogenized in a homogenizer with the addition of 10 mL of 95% ethanol. A primary ethanol extract containing all chloroplast pigments was obtained in this way. The extract was then centrifuged at 5000 r/min for 10 min. Since the concentration of pigments was high for reading by spectrophotometer, the obtained extract was diluted by adding 9 mL of 95% ethanol/mL of extract. The extract produced in this way was subjected to reading on a spectrophotometer.

2.5. Seedling fluorescent detection

During seed germination and seedling growth, the treated seeds were observed at 4d, 6d, 10d and 16d of germination, respectively. Photos were taken by a fluorescence microscope (Leica MZ16FA) with filter model (excitation wavelength: 480–560 nm; emission wavelength: 580–610 nm).

2.6. Standard curve drawing of rhodamine B

The standard solution of RB with concentration of 5.0, 10.0, 15.0, 25.0 mg/L were prepared, respectively. After spectrum scanning by UV-1800 type ultraviolet-visible spectrophotometer, the absorbance value at 554 nm was detected and the standard curve of RB was obtained. The linear regression equation was $A = 0.0239 + 0.1973C$, with the correlation coefficient $R = 0.9998$. As the curve has good linear relation, the residual concentration of RB in liquid was calculated according the absorbance value in the adsorption process.

2.7. The adsorption of rhodamine B by field soil

Standard solution of RB with concentration of 10, 15, 20, 25, 30, 35, 40, 45, 60, 70, 80 mg/L were prepared, respectively.

Confirming the saturated adsorption time: 50 mL RB solution (2.0 mg/L) was added 1.0 g field soil, and then the solution was stirred with a magnetic stirrer at 25 ± 2 °C with rotation speed 160 r/min. After the adsorption time of 1 h, 6 h, 12 h, respectively, supernatant were obtained through centrifugation separation and their absorbance value were detected by UV-1800 type ultraviolet-visible spectrophotometer, then the saturated adsorption time was confirmed according the absorbance value.

Determining the maximum adsorption of RB by soil: 50 mL RB solution (with different concentration) were obtained by measuring cylinder, and added 1.0 g field soil, respectively, then stirred with a magnetic stirrer at 25 ± 2 °C with rotation speed 160 r/min. After the saturated adsorption time, supernatant were obtained through centrifugation separation and their absorbance values were detected by UV-1800 type ultraviolet–visible spectrophotometer. The residual concentration of RB in liquid (C_t) was calculated according to the absorbance value in the adsorption process. The maximum adsorption of RB (q) by 1.0 g soil could be calculated by $q = (C_0 - C_t)\,V/m$. C_0 was the initial concentration of RB (mg/L); C_t was the concentration of RB in t time (mg/L); V was the volume of 1RB solution (L); m was the quality of field soil (g).

2.8. The photo degradation of RB absorbed by soil

1.0 g field soil was added to 50 mL RB solution (60 mg/L), after saturated adsorption, the soil become red color. The red soil was put to a beaker containing 50 mL solution with H_2O_2 (0.10 mol/L) and Fe^{2+} (2.33×10^{-4} mol/L), and was irradiated under visible light (UV/Fenton). The absorbance value of RB in saturated adsorption soil, pre-and post of UV/Fenton, was detected by UV-1800 type ultraviolet-visible spectrophotometer.

2.9. Statistical analysis

Statistical Analysis System (SAS) software was used to analyze means of analysis of variance (ANOVA) and multiple comparisons (LSD, $\alpha = 0.05$). Percentage data were transformed according to $y =$ arc sin [sqr (x/100)] before analysis (Hu et al., 2005).

3. Results

3.1. Effects of RB on seed germination and seedling growth

There were no significant difference in germination percentage, germination energy, vigor index, seedling dry weight, root length and seedling height after coating with 1:10 and 1:20 RB from the control coated with base coating agent. The vigor index, seedling dry weight and shoot height when coating with 1:30 RB were all significantly higher than those of non-coated control (Table 1). There were no significant differences in seedling protective enzymes, MDA and chlorophyll content among all treatments (Table 2).