Australian Journal of Crop Science AJCS
AJCS 7(1):147-151 (2013) ISSN:1835-2707

Application of fluorescent dyes for falsification-preventing of pea seeds (*Pisum sativum* L.)

Yixin Tian, Qitian Wang, Jin Hu*, Qijuan Hu, Jiancheng Wang, Yajing Guan*

Seed Science Center, College of Agriculture and Biotechnology, Zhejiang University, Hangzhou 310058, P.R. China

***Corresponding authors: jhu@zju.edu.cn, gyj02127@126.com**

Abstract

In the present study, combining with the technology of seed coating, the fluorescent compounds of rhodamine B (RB) and safranine T (ST) were used to label seeds as an anti-counterfeiting technology. The experiment was performed to investigate the effects of coating seeds with fluorescent dyes on physiological parameters of pea and the fluorescence performance in pea seedlings. The results showed that fluorescent dyes had no negative effect on seed germination, seedling growth, seedling protective enzyme activities, malondialdehyde (MDA) and chlorophyll content after coating with mass ratio of 1Kg RB to 20～30Kg pea seeds and 1 Kg ST to 10～30Kg pea seeds. RB under fluorescence microscope emitted bright red fluorescence and bright orange fluorescence excited by green light (546 nm) and blue light (495 nm), respectively. The fluorescence intensity in RB was higher than that in ST. Moreover, the vascular bundles of stem, roots and aerial parts of seedlings treated with RB all emitted brilliant fluorescence for a long time, which could be used as a marker in seedlings. It suggests that pea seeds labeling with RB dye by coating way at appropriate proportion can be used as an anti-counterfeiting technique in pea seeds.

Keywords: Anti- counterfeiting, fluorescence, fluorescent dyes, pea seed, seeding growth.
Abbreviations: POD_Peroxidase, CAT_Catalase, APX_Ascorbate Peroxidase, SOD_Superoxide Distamuse, MDA_ Malondialdehyde, RB_Rhodamine B, ST_Safranine T.

Introduction

Pea (*Pisum sativum* L.) is one of the most important legume crops in the world and covers a great part of world cereal market. The demand for high quality pea seed has been increased recently due to its value in food products, vegetables, feed and green manure etc. However, fake seeds have been found in market of developing countries, which not only causes great loss to farmers and seed corporations, but also influences negative effect on the yield and quality of crop (Guan et al., 2011). Many different methods have been used by seeds corporations to strengthen their brand protection, such as external package anti-counterfeiting technologies (Zhang et al., 2007b). However, due to reproducibility of the external package, it still could not effectively eliminate fake seeds in the market (Wang, 2009; Cai, 2009). Therefore, new anti-counterfeiting methods, which directly applied on seed, are needed urgently. In recent years, the fluorescent compounds are widely applied in clinical diagnosis, food, and the analysis of physical and chemical compounds, which make it possible to be used as anti-counterfeiting indicators. It was reported that the distribution of hem agglutinin in root tips could be determined using the fluorescence performance of rhodamine in root (Hapner, 1978). In another study, propagation characteristics of pollen could be directly observed and studied with fluorescent dyes as mimics of pollen (Nickolas and Mary, 1982). The experiment of the distribution of mitochondria of cabbage apical in the process of dehydration using rhodamine 123 indicated that the mitochondria gradually congealed to form a clot and then the circulation of cytoplasm stopped (Wu, 1987). The study of the movement of water into wheat kernels using fluorescent dyes showed that the water entered to the wheat germ first, then ventral groove and finally extended to the end of hairs along the nucellar layer and aleuronic layer (Shi et al., 2001). Fluorescent indicator HPTS (8-hydroxy, 3, 6- three acid pyrene) was successfully used to trace the route of nutrients into the seed coat (Joost and Amkie, 2003). Many works have been carried out using fluorescent compounds as indicators in plant research. Only very limited works have been reported regarding the successful use of fluorescent compounds for seed anti-counterfeiting except safranine T (ST) used as a label in tobacco seeds (Guan et al., 2011). There are differences in the structure and permeability of different crops seeds. We need to understand whether other fluorescent dyes could be used as anti-counterfeiting labels in different seeds. In addition, the seed coating and seed dressing agents, marked with its high technology and economic benefits, is an advanced comprehensive technique, which will ensure an impetus to a healthy growth of the seed industry. In the present study, the pea seeds and different fluorescent dyes were used as experimental materials and indicators. The optimal fluorescent indicator as an anti-counterfeiting label was selected according to the influence of fluorescent dyes on seed germination and seedling growth. The dynamic characteristics of fluorescent indicators in pea seedling were also investigated.

Table 1. Effects of coating seeds with RB on pea seed germination and seedling growth.

Treatments (RB/seed)	GP (%)	GI (%)	VI	DW (g/20 plants)	RL (cm)	SH (cm)
CK	95.33a*	95.33a	26.25a	0.50a	8.54ab	5.18b
CK1	96.67a	96.67a	26.19a	0.46a	8.99a	6.04ab
CK2	95.33a	95.33a	26.79a	0.47a	9.23a	6.13ab
CK3	92.00a	92.00a	27.20a	0.48a	9.09a	6.13ab
1:10	92.00a	92.00a	24.20b	0.48a	7.55b	4.99b
1:20	94.67a	94.67a	26.78a	0.48a	9.42a	6.46ab
1:30	96.67a	96.67a	26.92a	0.48a	9.45a	7.67a*

*****Significant difference ($p<0.05$, LSD) among treatments. The GP, GI, VI, RL, SH and DW mean germination percentage, germination index, vigor index, root length, shoot height, seedling dry weight, respectively. The CK, CK1, CK2 and CK3 mean control non-coated, control coated with the mass ratio of base coating agent without RB to pea seeds 1:10, 1:20, 1:30, respectively. 1:10, 1:20 and 1:30 mean coated with the mass ratio of coating agent with RB to pea seeds 1:10, 1:20 and 1:30, respectively.

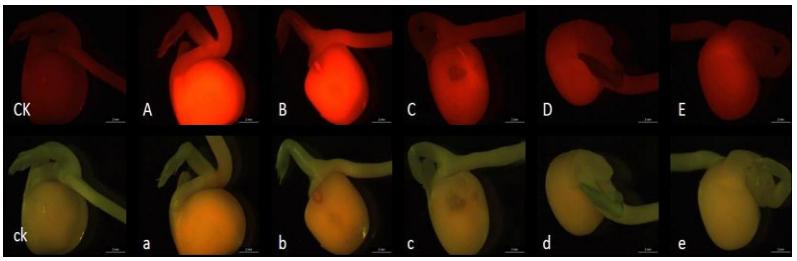

Fig 1. The fluorescence of pea seeds coated with RB and ST, respectively on 4th day of germination. The CK~E, means the fluorescence under green light (546nm). The ck~e, means the fluorescence under blue light (495nm). A and a, mean the fluorescence of seeds coated with the mass ratio of coating agent with RB to pea seeds 1:20. B and b, mean the fluorescence of seeds coated with the mass ratio of coating agent with RB to pea seeds 1:30. C and c, mean coated with the mass ratio of coating agent with ST to pea seeds 1:10. D and d, mean the fluorescence of seeds coated with the mass ratio of coating agent with ST to pea seeds 1:20. E and e, mean the fluorescence of seeds coated with the mass ratio of coating agent with ST to pea seeds 1:30. CK and ck, mean the fluorescence of seeds non-coated.

Results

Effects of fluorescent indicators on seed germination and seeding growth

Seed germination percentage, germination energy, seed vigor index, seedling dry weight, root length and seedling height, treated with various coating ratio of the fluorescent compounds RB, were not significantly different from the control coated with base coating agent. The VI, root length and shoot height coated with 1:10 RB were significantly decreased (Table 1). Meanwhile, there were no significant differences among treatments treated with various coating ratios of ST from the control coated with base coating agent (Table 2). Shoot height coated with 1:30 RB and 1:30 ST were all significantly higher than non-coated control (Table 1 and 2). In pea seedling, the decrease of POD and SOD activity and increase of MDA content, coated with 1:10 RB, reached a significant level compared with the control coated with base coating agent. Activities of APX and SOD were significantly increased and MDA content was significantly declined in coated with 1:30 RB, when compared with the control non-coated treatment. Besides, there were no significant differences in seedling protective enzymes, MDA and chlorophyll content among treatments (Table 3). Seedling protective enzymes, MDA and chlorophyll content treated with various coating ratio of ST had no significant influence compared with the control of the same coating ratio, and the increase of POD and SOD activity and decrease of MDA content in coated with 1:30 ST reached a significant level, compared to the control non-coated seedling (Table 4).

Detection of fluorescence performance in seedling

The results showed that RB (coated with 1:20 and 1:30) and ST (coated with 1:10, 1:20 and 1:30) all have improved pea seedling establishment to some extent. Therefore, they were used as fluorescent indicators. Their fluorescence was observed under fluorescence microscope in seedlings. The treated seedlings (especially with RB) showed red fluorescence under green light excitation, when compared with the non-coated control (Fig 1, A, B), and bright orange fluorescence under blue light (Fig 1, a, b). Although seedlings, treated with ST, presented red and orange fluorescence when excited by green and blue light, respectively, there was no obvious difference between the treatment and the control (Fig 1, C~E, c~e).

Seedling fluorescence detection after RB treatment

The whole pea seedlings, treated with RB, showed obvious red fluorescence under green light (Fig 2, Z-2, Z-4, Z-7, Z-9) and orange fluorescence under blue light (Fig 2, z-2, z-4, z-7, z-9) on the 2th, 4th, 7th and 9th day of seed germination, compared with the fluorescent of the control seedling (Fig 2, CK-2, CK-4, CK-7, CK-9). Along with the seedling growth, red and orange fluorescence of pea seeds gradually weakened, but still showed visible differences from the control. After growing for 9th day, a whole seedling could not be observed in a vision of microscope, so it was divided into three parts (cotyledon, stem and root) for observation.

Table 2. Effects of coating seeds with ST on pea seed germination and seedling growth.

Treatments (ST/seed)	GP (%)	GI (%)	VI	DW (g/20 plants)	RL (cm)	SH (cm)
CK	95.33a*	95.33a	26.25a	0.50a	8.54a	5.18b
CK1	96.67a	96.67a	26.19a	0.46a	8.99a	6.04ab
CK2	95.33a	95.33a	26.79a	0.47a	9.23a	6.13ab
CK3	92.00a	92.00a	27.20a	0.48a	9.09a	6.13ab
1:10	93.33a	93.33a	25.32a	0.50a	8.83a	6.85a
1:20	97.33a	96.67a	26.37a	0.49a	10.18a	7.38a
1:30	98.00a	97.33a	26.73a	0.51a	9.85a	7.36a*

*Significant difference ($p<0.05$, LSD) among treatments. The GP, GI, VI, RL, SH and DW mean germination percentage, germination index, vigor index, root length, shoot height, seedling dry weight, respectively. The CK, CK1, CK2 and CK3 mean control non-coated, control coated with the mass ratio of base coating agent without ST to pea seeds 1:10, 1:20, and 1:30, respectively. The 1:10, 1:20 and 1:30 mean coated with the mass ratio of coating agent with ST to pea seeds 1:10, 1:20 and 1:30, respectively.

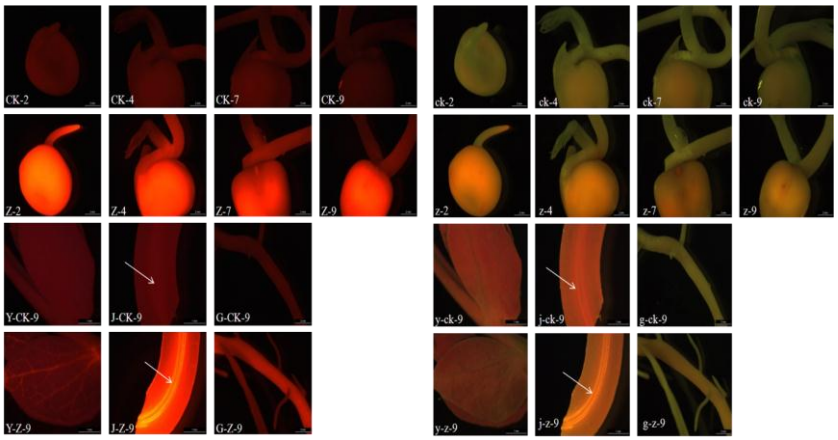

Fig 2. The fluorescence of pea seedling coated by agent with RB (mass ration 1:20) under the green light (546 nm, expressed by uppercase letters) and blue light (495 nm, expressed by lowercase letters), respectively. The CK-2, CK-4, CK-7 and CK-9, mean the fluorescence of seed non-coated on the 2th, 4th, 7th and 9th day germinated, respectively. The Z-2, Z-4, Z-7 and Z-9, mean the fluorescence of seed coated with RB agent on the 2th, 4th, 7th and 9th day germinated, respectively. The Y-CK-9, J-CK-9 and G-CK-9, mean the fluorescence of cotyledon, stem and root non-coated on 9th day germinated, respectively. The Y-Z-9, J-Z-9 and G-Z-9, mean the fluorescence of cotyledon, stem and root coated with RB agent on 9th day germinated, respectively. The lowercase letters (corresponding to uppercase letters) mean the fluorescence of the same place of seedling under blue light. White arrows mean the vascular bundles of stem.

A brilliant red fluorescence was found in the veins of cotyledon under green light on the 9th day, compared to control seedling (Fig 2, Y-Z-9, Y-CK-9). However, no obvious different was observed under blue light, compared to control (Fig 2, y-z-9, y-ck-9). Root had the similar fluorescence performance to cotyledon (Fig 2, G-Z-9, G-CK-9 and Fig 2, g-z-9, g-ck-9). Bright yellow and orange fluorescence was found in the vascular bundle of stem (Fig 2, J-Z-9, J-CK-9 and Fig 2, j-z-9, j-ck-9), and the intensity was higher than that in root and cotyledon at the same day.

Discussion

Nowadays, application of fluorescent compounds as anti-counterfeiting in plant seeds has been considered as a novel approach. However, greater concern is the phytotoxicity of these materials to seed and plant system, which limits their wide scale applications (Remya et al., 2011). In addition, the technology of seed coating is much more efficient and safe than traditional seed treatment technologies such as soaking (Song et al., 2004). So, combining with the technology of seed coating, present experiment studied the effects of RB and ST with different coating ratios on seed germination and seedling growth for optimizing their concentration at a safe level. Enzymatic mechanism includes cooperation of protective enzymes, such as SOD, POD, CAT, APX which could be used as indicators of the vigor of seeds and seedlings (Zhang et al., 2007a). Also, MDA is a major component of thiobarbiturate-reactive substances and is used as an indicator of lipid peroxidation (Zhang et al., 2001). Besides, chlorophyll is related to the normal growth and development of plant, which is an important physiological index of photosynthesis in plant (Zhang et al., 2007b). In the present paper, the results suggested that the protective enzymes activities, the content MDA and chlorophyll were not negatively affected by RB and ST, under a lower coating ratio. In contrast, the pea seedling establishment was improved to some extent. There are similar reports in tobacco seeds and marigold (Guan et al., 2011; Sellei, 1941). Besides, after detecting the fluorescence in seedling, the results showed that the red fluorescence of RB was stronger than that of ST and had evident difference from the control. Therefore, RB was considered more suitable for seed anti-counterfeiting. RB could present brilliant red and distinct orange fluorescence when excited by green and blue light, respectively. The effective transportation of RB was observed in seed and seedling of pea through the tracking of fluorescence performance of RB treated seed. However the fluorescent gradually weakened with the growth of seedling. The veins of cotyledon, the vascular bundles of stem and the roots presented strongest fluorescence even seedling grew for 9 days under green light. It seems that the fluorescent dye could be absorbed by root and transported up to leaves through the vascular bundles of stem along with seed germination. The reason remains to elucidate.

Table 3. Effects of coating seeds with RB on protective enzyme activities, MDA and chlorophyll content of pea seedlings.

Treatments (RB/seed)	POD (nmol·min-1· g-1 FW)	CAT (nmol·min-1· g-1 FW)	APX (nmol·min-1· g-1 FW)	SOD (μ/g)	MDA (nmol/g)	Chlorophyll (mg/g)
CK	60.39d*	2.11a	7.89b	439.88c	40.31b	0.85a
CK1	60.78d	2.29a	8.31ab	440.92c	39.70bc	0.87a
CK2	66.62c	2.33a	8.32ab	443.38b	39.96bc	0.89a
CK3	67.68ab	2.17a	9.29ab	443.50b	39.45bc	0.91a
1:10	58.32e	1.68a	8.09ab	438.01d	42.11a	0.87a
1:20	67.11bc	2.07a	8.42ab	445.32a	38.93bc	0.93a
1:30	68.19a*	2.34a	9.88a	445.83a	38.43c	0.97a

*Significant difference ($p<0.05$, LSD) among treatments within the same fluorescent indicator; POD, CAT, APX, SOD and MDA mean peroxidase, catalase, ascorbate peroxidase, superoxide distamuse, malondialdehyde respectively; CK, CK1, CK2 and CK3 mean control non-coated, control coated with the mass ratio of base coating agent without RB to pea seeds 1:10, 1:20, 1:30, respectively; 1:10, 1:20 and 1:30 mean coated with the mass ratio of coating agent with RB to pea seeds 1:10, 1:20 and 1:30, respectively.

Table 4. Effects of coating seeds with ST on protective enzyme activities, MDA and chlorophyll content of pea seedlings

Treatments (ST/seed)	POD (nmol·min^{-1}·g^{-1} FW)	CAT (nmol·min^{-1}·g^{-1} FW)	APX (nmol·min^{-1}·g^{-1} FW)	SOD (u/g)	MDA (nmol/g)	Chlorophyll (mg/g)
CK	60.39c*	2.11a	7.89a	439.89b	40.31a	0.85a
CK1	60.78c	2.29a	8.31a	440.92b	39.70a	0.87a
CK2	66.62b	2.33a	8.32a	443.38a	39.96a	0.89a
CK3	67.68ab	2.17a	9.29a	443.50a	39.45ab	0.91a
1:10	60.22c	1.91a	7.75a	440.24b	39.96a	0.86a
1:20	67.85ab	2.23a	8.20a	442.98a	38.97ab	0.92a
1:30	68.19a	2.31a	8.66a	443.61a	38.06b	0.99a

*Significant difference ($p<0.05$, LSD) among treatments within the same fluorescent indicator; POD, CAT, APX, SOD and MDA mean peroxidase, catalase, ascorbate peroxidase, superoxide distamuse, malondialdehyde respectively; CK, CK1, CK2 and CK3 mean control non-coated, control coated with the mass ratio of base coating agent without ST to pea seeds 1:10, 1:20, 1:30, respectively; 1:10, 1:20 and 1:30 mean coated with the mass ratio of coating agent with ST to pea seeds 1:10, 1:20 and 1:30, respectively.

Anti-counterfeiting fluorescent label could be observed in different parts of seedling, when compared with external anti-counterfeiting package technologies. The effective time was also prolonged for seed anti-counterfeiting. Moreover, because of the specific fluorescent color and the special wavelength of excitation light, this technology could not be imitated easily. Meanwhile, fluorescent marker could be fast and easily detected by testing equipments, which was very beneficial to users. Since some fluorescent compounds have proved to be safe for plants and showed good fluorescence in pea seed and seedlings, their use for anti-counterfeiting labeling on seed is highly recommended. We have checked the stability of fluorescent in pea seedlings, and our results proved good stability of fluorescence from RB with a long period time. Fluorescent compounds exist photo bleaching, and the decomposition rate of them may be affected by different environments, which may affect fluorescence labeling time. Hence, further studies need focusing on more plant species and effective fluorescence dyes for the successful wider application in anti-counterfeiting using.

Materials and methods

Plant materials

Pea seeds, cv. ZhongWan 4, from Lvwawa Seed Company, Hangzhou, P. R. China, were used as experimental materials. Base seed coating agent obtained from Seed Science Center of Zhejiang University, Hangzhou, P. R. China. Fluorescent dyes, rhodamine B (RB) and safranine T (ST) were obtained from Aladdin Company, Hangzhou, P. R. China. Seed coating agent were obtained from base coating agent added with RB (500mg/L) and ST (500mg/L), respectively.

Seed labeling

Pea seeds were coated with two types of seed coating agents, including RB and ST separately, in which the mass ratio of coating agent to pea seeds were 1:10, 1:20, 1:30, respectively. Meanwhile, pea seeds coated with base seed coating agent (without adding RB and ST) 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 pea seeds were also used as a control, expressed by CK.

Seed germination and seedling growth

After labeling, 100 seeds were placed in a germination box with three layers of wetted blotters. Each of the three replicates was comprised of 100 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 8 days and the germinated seeds were recorded daily. Germination energy and percentage was calculated on the 5th and 8th day, respectively (ISTA, 2004). After germination for 8 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., 2007a). The germination index (GI = ∑(Gt/Tt)) and vigor index (VI= GI×Seedling height) 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 ∑ is the sum.

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 Hu et al. (2005). The chlorophyll content was determined according to Zhang et al. (2007a). The fresh seedling was 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 per ml of extract. The extract produced in this way was subjected to reading on a spectrophotometer.

Seedling fluorescent detection

During seed germination and seedling growth, the treated seeds were observed at 2d, 4d, 7d and 9d 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).

Statistical analysis

Experimental design of RCBD with three replications was conducted. Statistical Analysis System (SAS) software was used to analyze means of analysis of variance (ANOVA) and multiple comparison (LSD, $p<0.05$). Percentage data were transformed according to y =arcsin [sqr (x/100)] before analysis (Hu et al., 2005).

Conclusions

Our data suggests that pea seeds coated with suitable level of RB and ST can effectively serve as a falsification-preventing tool according to the fluorescence performance of seed and seedling. We recommend that, RB and ST are applicable as anti-counterfeiting for the limitation of traditional technology of with seed external packing and can be considered as a novel approach.

Acknowledgements

The research is supported by key project of Natural Science Foundation of Zhejiang Province (No. Z3100150) and Special Fund for Agro-scientific Research in the Public Interest (201203052), P. R. China.

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