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UPTAKE PATHWAYS OF FLUORESCENT INDICATORS BY PEA SEED AND SEEDLINGS AND THEIR POTENTIAL AS ANTI-COUNTERFEITING LABELING FOR PLANT SEEDS

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Abstract

The present study investigates the effects of seed soaking in varying concentration of rhodamine B (RB) or safranine T (ST) solutions on germination and seedling growth of pea seeds. The fluorescence in pea seedling at different developmental stages was observed. The results indicate that there were no adverse effects of seed soaking in RB (0.1mg/ml) and ST (0.5, 0.3, 0.1mg/ml) solutions on germination, seedling growth, antioxidant enzyme activities, malondialdehyde (MDA) and chlorophyll contents. The seeds treated with RB showed bright red and orange fluorescence under green (546 nm) and blue (495 nm) light excitation, respectively while no red or orange color was observed in the control seeds. In addition, the vascular bundles of stem, seedling roots and aerial parts of seedlings treated with RB all emitted brilliant fluorescence for a longer time as compared with that treated with ST. It can be concluded that pea seed labeled with RB by seed soaking at appropriate concentration could be used as a potential anti-counterfeiting technique in pea seeds.

Key words: Anti-counterfeiting, Fluorescence, Pea, Rhodamine B, Safranine T, Seeding growth.

Introduction

Pea (*Pisum sativum* L) is an important legume crop and widely grown in many parts of world. Due to the gradual increase in pea utilization as food, vegetables, feed and green manure etc, the demand for pea seeds with high quality increased. However, the fake seeds found in seed market result in a great loss of crop production and seed corporations (Zhou & Chen, 2005; Tian *et al.,* 2000). Although most of seed enterprises do their best to eliminate fake seed (Ahmad *et al.,* 2014; Wu *et al*., 2014), it is difficult to be accomplished just though some kinds of seed anti-counterfeit packaging technologies (Wang, 2009; Cai, 2009). Therefore, the new anti-counterfeiting methods applied on seed are urgently needed.

Since fluorescent compounds have been widely applied in clinical diagnosis and physical and chemical analysis recently, their characteristics of high stability and easily operation gained increasing attention, and make them possible to be used as potential anti-counterfeiting indicators. It was reported that the distribution of hemagglutinin in root tips could be determined through the fluorescence performance of rhodamine (Hapner, 1978) and the propagation characteristics of pollen could be directly observed with fluorescent dyes mimicking pollen (Nickolas & Mary, 1982). Moreover, 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 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). In addition, fluorescent indicator HPTS (8-hydroxy, 3, 6 - three acid pyrene) was successfully used to trace the route of nutrients into the seed coat (Joost & Amkie, 2003). It was found that fluorescein could improve the root performance and promote plant growth and development (Sellei *et al.,* 1942; Sellei, 1940; Li *et al.,* 2011). Although many works have been carried out using fluorescent compounds as indicators in plant research, very limited works have been reported regarding the successful use of fluorescent compounds for seed anti-counterfeiting except that safranine T (ST) was used as a label in tobacco seeds (Guan *et al.,* 2011). However, as the differences existed in the structure and permeability of different crops, whether other fluorescent dyes could be applied as seed anti-counterfeiting labels still remain to be explored.

Seed soaking is one of the most economical approaches for improving seed establishment in the field. Seeds could absorb fluorescent dyes through seed soaking in Rhodamine B (RB) and ST solutions. Meanwhile, when exposed to different excitation lights, RB and ST showed different fluorescent colors which were different from the fluorescence of plant itself. Therefore, RB and ST were used to label seed as a unique "fingerprint" in this study. Moreover, phytotoxic studies are important for the successful application of any fluorescent indicators to plant system. Many unfortunate consequences in plants have been caused by chemical treatments of seed. Protective enzymes, such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) can be used to indicate the vigor of seeds and seedlings (Zhang *et al.,* 2007a). Malondialdehyde (MDA) is a major component of thiobarbiturate-reactive substances, and reflects the level of lipid super oxidation action on cell membrane and the response of plants to some adverse conditions such as phytotoxicity (Zhang *et al.,* 2001). Besides, chlorophyll is an important physiological index of photosynthesis in plant, which is relation to normal growth and development of plant (Zhang *et al.,* 2007b). Therefore, these protect enzyme activities, MDA and chlorophyll content might be used as physiological markers of RB and ST treatment in pea seeds.

In the present study, the optimal fluorescent indicator was selected out according to the influence of fluorescent dyes on seed germination and seedling growth. Moreover, the uptake of fluorescent indicators by seed and the dynamic characteristics of fluorescent indicator in pea seedling were also investigated.

Material and Methods

Materials: Pea seeds, cv. ZhongWan 4, from Lvwawa Seed Company, Hangzhou, P. R. China, were used as experiment materials. Fluorescent dyes, rhodamine B (RB) and safranine T (ST) were obtained from Aladdin Company, Hangzhou, P. R. China.

Seed labeling: The fluorescent solutions of RB and ST with different concentrations (0.1, 0.3 and 0.5 mg/ml) were prepared with deionized water respectively. Then, 100g pea seeds were soaked in 500ml fluorescent solutions respectively, for 24 h at 20^{o}C in dark. Then, the residues of fluorescent dyes on seed coat were rinsed by distilled water. The seeds soaked in water were used as a control. Treated and control seeds were dried back to their initial moisture content (7.0%).

Seed germination and seedling growth: After labeling, 100 seeds were placed in a germination box with four layers of wetted blotters. Each of the three replicates was comprised of 100 seeds. Then germination boxes were incubated in a growth chamber (GZH-268B, HangZhou) under alternative cycle of 12 h light and 12 h darkness at 20^{o}C for 8 days and the germinated seeds were recorded daily (Anon., 2004). Germination energy (GE) and percentage (GP) was calculated on the 5th and 8th day, respectively. The vigor index (VI=$\sum$(Gt/Tt)×seedling height) was 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 $\sum$ is the sum. Root length (RL), shoot height (SH) and dry weight (DW, weighed after drying at 80^{o}C for 72 h) were manually measured on twenty randomly selected seedlings from each replicate after growing for 8 days.

Detection of seedling protective enzymes, MDA and chlorophyll content: The activity of peroxidase (POD) in seedling leaves was determined by guaiacol method (Qiu *et al.,* 2005). Activities of catalase (CAT), ascorbate peroxidase (APX) and superoxidase dismutase (SOD) in seedling leaves were determined by the method described by Cao *et al.* (2010). The content of malondialdehyde (MDA) in seedling leaves was measured using thiobarbituric acid reaction method (Gao *et al.,* 2009). The total chlorophyll content was calculated according to Zhang *et al.* (2007a). All measurements mentioned above were made after seed germination for 8 days. The absorbance changed 0.01 in one minute as 1 unit of the enzyme activity (U): U/gFW·min.

Seed and Seedling fluorescent detection: After soaking in fluorescent indicators for 6, 12, 18, 24and 30 h, seeds were cut in half by a freezing microtomy and were observed, respectively by a fluorescence microscopy (Leica MZ16FA) with filter model (excitation wavelength: 480-560 nm; emission wavelength: 580-610 nm). During seed germination and seedling growth, the treated seeds and seedlings were observed at 2, 5, 8 and 12 d, respectively. Micrographs of the fluorescence from the fluorescein in these seedlings were taken by camera (Leica DFC420C) and the photos of the fluorescence excited by green light (530-560 nm) and blue light (480-500nm) were also been taken.

Statistical analysis: The data were subjected to an analysis of variance (ANOVA) with Statistical Analysis System (SAS) software, and when a significant (p<0.05) F ratio occurred for treatment effects, a least significant difference (LSD) was calculated. Before ANOVA, the data of percentage were transformed according to y =arcsin [sqr (x/100)].

Results

Effects of fluorescent dyes on pea seed germination and seeding growth: Seed treatment with 0.1mg/ml of RB maximally germination percentage and seedling vigour as indicated by higher RL, SH and DW (Table 1). However, 0.5mg/ml of RB significantly decreased the seed GP, GE, VI, RL, SH and DW. Meanwhile, the VI of seeds treated with ST of three concentrations was all significantly higher than the control (Table 1). And there were no significant differences in GR, GE, DW, RL and SH among ST treatments and the control.

The MDA content of pea seedling reduced significantly by 0.1mg/ml of RB (Table 2). In addition, for protective enzyme activities, there were no significant differences among treatments irrespective of fluorescent dyes, except that the POD was significantly decreased by 0.5 mg/ml of RB. The APX was significantly increased by all treatments of ST in comparison with the control (Table 2). Meanwhile, the chlorophyll content significantly increased and reduced after treated with 0.1 and 0.5 mg/ml of RB, respectively.

Detection of fluorescence showing in pea seedling: The seedling treated with RB and ST showed obviously red fluorescence (Fig. 1, A~D) under green light excitation and revealed bright orange fluorescence (Figs. 1, a~d) under blue light illumination as compared with the control (Figs. 1, CK, ck). In addition, pea seedling showed brighter fluorescence with RB than ST of the same concentration (Fig. 1, A, D, a, d). Considering the better fluorescence and cheaper cost in RB than those of ST, RB was selected for further fluorescence study of pea seedling.

Absorption of RB by pea seed: Pea seed soaked in RB solution showed brilliant yellow (Fig. 2, A~E) and orange fluorescence (Fig. 2, a~e) under green and blue light excitation separately, while on such fluorescence was observed in the control seed (Fig. 2CK, 2ck). After seed soaking for 6 h, fluorescent signals appeared in peripheral region of cotyledon near to seed coat (Fig. 2, A, a). With soaking time increasing, fluorescent dye infiltrated slowly from periphery to inner of cotyledon and formed a ring zone with brilliant fluorescence.

Table 1. Effects of fluorescent indicators on pea seed germination and seedling growth.

Fluorescent dye	Treatments (mg/ml)	GP (%)	GE (%)	DW (mg/plant)	RL(cm)	SH(cm)	VI
RB	CK	96.00a*	96.00a	31.50a	12.81a	5.85b	29.01a
	0.1	97.33a	97.33a	30.50a	12.66a	8.20a	39.67a
	0.3	93.33ab	93.33ab	24.50ab	6.68b	4.76bc	22.52ab
	0.5	81.33b	81.33b	20.00b	3.11b	3.24c	15.37b
ST	CK	96.00a	96.00a	31.50a	12.81a	5.85a	29.01b
	0.1	94.00a	94.00a	31.50a	14.06a	5.94a	46.17a
	0.3	92.00a	92.00a	33.00a	11.97a	7.30a	44.90a
	0.5	93.33a	93.33a	29.50a	13.12a	7.53a	46.50a

*Significant difference ($\alpha<0.05$, LSD) among treatments within the same fluorescent indicator; GP, GE, VI, RL, SH and DW mean germination percentage, germination energy, vigor index, root length, shoot height, dry weight, respectively; RB and ST mean rhodamine B, safranine T, respectively

Table 2. Effects of fluorescent indicators on protective enzyme activities s, MDA and chlorophyll content of pea seedlings.

Fluorescent indicators	Treatments (mg/ml)	POD ($nmol \cdot min^{-1} \cdot g^{-1}$ FW)	CAT ($nmol \cdot min^{-1} \cdot g^{-1}$ FW)	APX ($nmol \cdot min^{-1} \cdot g^{-1}$ FW)	SOD ($u \cdot g^{-1}$)	MDA ($nmol \cdot g^{-1}$)	Chlorophyll content ($mg \cdot g^{-1}$)
RB	CK	94.44a*	1.43a	4.95a	313.20a	58.01a	0.66b
	0.1	101.16a	1.81a	5.10a	315.97a	36.82b	0.91a
	0.3	97.22a	1.34a	4.73a	275.34a	44.76ab	0.64b
	0.5	24.12b	0.90a	4.20a	249.21a	56.66a	0.32c
ST	CK	94.44a	1.43a	4.95b	313.20a	58.01a	0.66a
	0.1	95.88a	1.53a	6.19a	316.69a	54.15a	0.65a
	0.3	94.02a	2.65a	6.21a	316.26a	54.05a	0.87a
	0.5	96.28a	2.23a	6.11a	309.05a	50.48a	0.68a

*Significant difference ($\alpha<0.05$, LSD) among treatments within the same fluorescent indicator; POD, CAT, SOD and MDA mean peroxidase, catalase, superoxide distamuse, malondialdehyde, respectively; RB and ST mean rhodamine B and safranine T

Seedling fluorescence detection after RB treatment: On the 2th day, the whole pea seedling treated with RB showed obvious red fluorescence under green light (Fig. 3, Z-2) and orange fluorescence under blue light (Fig. 3, z-2) as compared with the control (Fig. 3, Z-CK-2, z-ck-2). After growing for another two days, the seedling could not be observed entirely in a vision of microscope, so it was divided into four parts (seed, cotyledons, stem and root) for observation.

For pea seed, its red (Figs. 3, Z-2, Z-5, Z-8, Z-12) and orange fluorescence (Figs. 3, z-2, z-5, z-8, z-12) gradually weakened with the seedling growth, but still showed visible differences from the control.

The 6-day-old cotyledon treated with RB showed obvious red fluorescence under green light (Fig. 3, Y-6). When cotyledons were magnified under microscope, bright red fluorescence also could be found in the vascular on the 8th day (Fig. 3, Y-8) and 12th day (Fig. 3, Y-12), respectively. However, under blue light, fluorescence in cotyledons (Figure 3, y-6, y-8, y-12) had no obvious differences from the control (Fig. 3, y-ck-6, y-ck-8, y-ck-12).

Seedling stem had the similar fluorescent performance to those showed in cotyledons. The fluorescence under green light (Figs. 3, J-6, J-8, J-12) in stem were stronger than that under blue light (Fig. 3, j-6, j-8, j-12), and gradually weakened with seedling growth. Moreover, fluorescence in stem top disappeared faster than those in stem bottom. In the longitudinal sections, bright yellow (Fig. 3, J-H-8, J-H-12) and orange fluorescence (Fig. 3, j-h-8, j-h-12) were found in the vascular bundle of stem on the 8th day and 12th day, respectively.

The root treated with RB also exhibited obvious red (Fig. 3, G-8, G-12) and orange fluorescence (Fig. 3, g-8, g-12) under different lights in comparison with the control.

Discussion

Using fluorescent compounds as seed anti-counterfeiting indicators have been considered as a novel approach to enhance seed security recently. However, the fluorescent chemicals must have no toxic to seed germination and seedling growth; otherwise it can't be applied in practice (Remya *et al.,* 2011). According to present findings, 0.1mg/ml for RB and 0.1~0.5 mg/ml for ST were considered as optimal concentrations for pea seed soaking due to their no adverse effect on seedling performance and even improved pea seedling establishment to some extent. Moreover, RB could present brilliant red and distinct orange fluorescence when excited by green and blue light, respectively. And the fluorescence of RB was apparently stronger than that of ST in equal concentration by detecting the fluorescence in seedling. Therefore, RB was considered more suitable for seed anti-counterfeiting.

After soaking in RB solution, the inside of seed showed well-distributed fluorescence under different excitation lights, but no such phenomenon existed in the control seeds. These observations indicated that the seed coat of pea was permeable to RB. Moreover, the stronger fluorescence appeared in hypocotyls may be contributed to the short distance between hypocotyls and micropyle.

Through tracking the fluorescence performance of RB treated seed, the effective transportation of RB was also observed in seedling. Although the fluorescence gradually weakened with the growth of seedling, cotyledons, the vascular bundles of stem and the roots all present bright fluorescence, and even the 12-day-old seedling did. The fluorescence was stronger under green light than blue light, and the fluorescence inside of a seed was brilliant yellow color, whereas that in seedling was bright red color, that might result from the diverse dyes and different plant tissues.

Therefore, there might be two uptake pathways of RB in pea seed, one was through the seed coat to the seed inside and the other was through emerging seedling tissues after germination occurred. The fluorescent tracer was up-taken by the emerging radical and move up to the above ground portion of seedlings. However, the uptake pathway warrants further study.

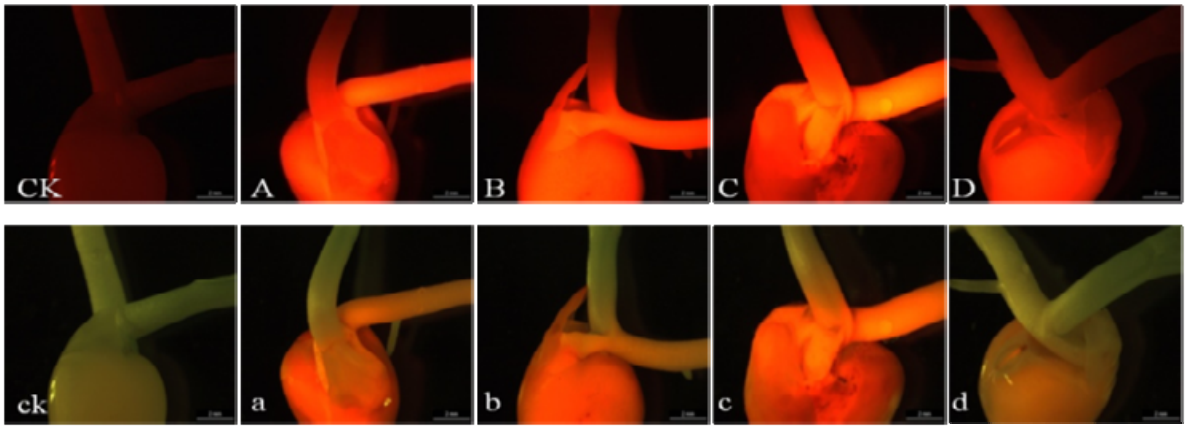

Fig. 1. The fluorescence of pea seeds soaked in RB or ST solutions of different concentrations on 6th day germinated (×7, bar=2mm). **CK~D**, uppercase letters, mean the fluorescence under green light (546nm). **ck~d**, lowercase letters, mean the fluorescence under blue light (495nm).**A** and **a**, seeds treated with 0.1mg/ml RB; **B** and **b**, seeds treated with 0.5mg/ml ST; **C** and **c**, seeds treated with 0.3mg/ml ST; **D** and **d**, seeds treated with 0.1mg/ml ST. **CK** and **ck**, seeds treated without RB or ST.

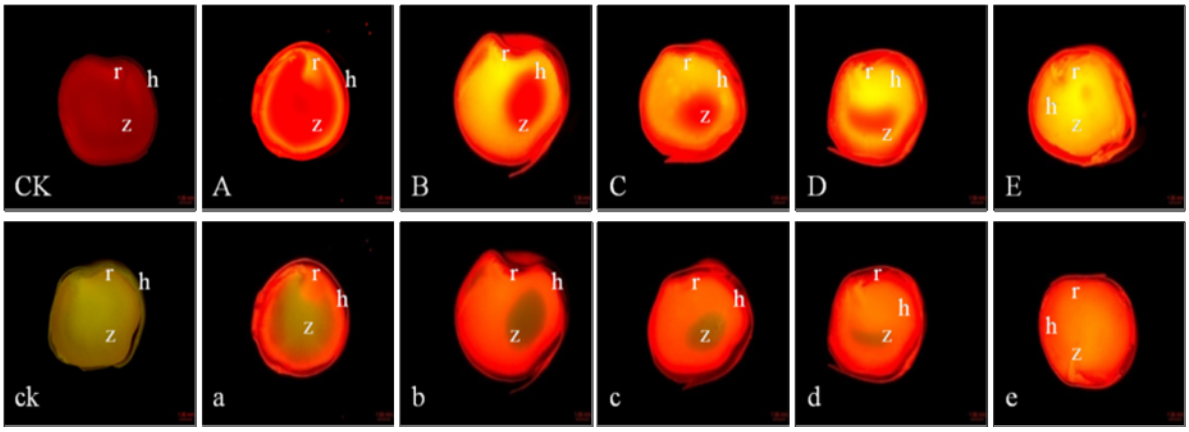

Fig. 2. The absorption of 0.1mg/ml RB by pea seed at different soaking time (bar=0.517). **CK~E**, uppercase letters, mean the fluorescence under green light (546nm). **ck~e**, lowercase letters, mean the fluorescence under blue light (495nm). **A** and **a**, the fluorescent signal in seed soaked for 6h; **B** and **b**, the fluorescent signal in seed soaked for 12h; **C** and **c**, the fluorescent signal in seed soaked for 18h; **D** and **d**, the fluorescent signal in seed soaked for 24h. **E** and **e**, the fluorescent signal in seed soaked for 30h, **CK** and **ck**, seeds treated without RB; **r, h, z** mean radical, hypocotyl and cotyledon, respectively.

Conclusions

Present results revealed that anti-counterfeiting fluorescent label of RB could be observed in different parts of seedling. And this technology could not be imitated easily due to the specific fluorescent color, specific wavelength of excitation light, suitable application amount of dyes and so on. In addition, it was very beneficial to users for faster and easier fluorescent marker detection with simple testing equipment.

Different fluorescent dyes have different fluorescent characteristics (Lavisand Raines, 2008). When selecting most efficient fluorescent dyes and labeling methods, the performance of fluorescent materials, the seed absorption characteristics, the seed processing technique and even the cost of treatment should all been taken into account (Salanenka & Taylor, 2006). Further studies needed focusing on more plant species and effective fluorescence dyes for the successfully wider application in seed anti-counterfeiting.

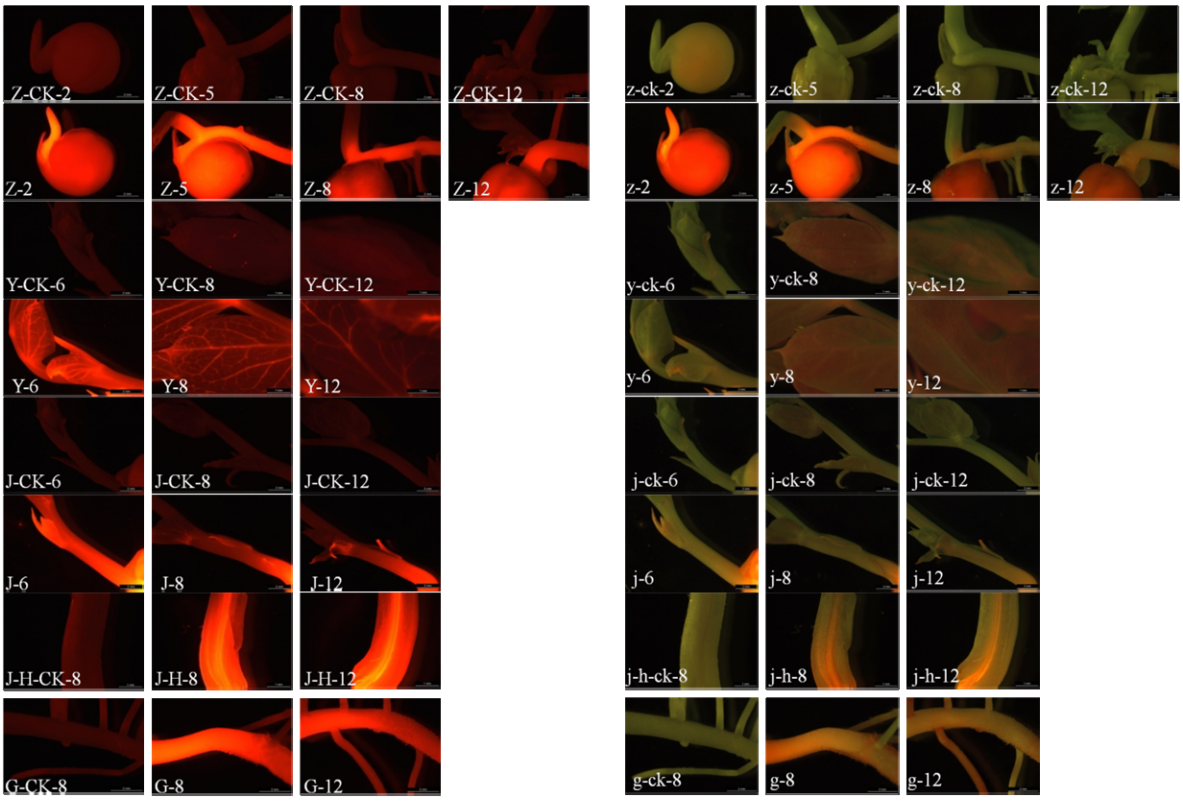

Fig. 3. The fluorescence in different parts of pea seedling under green (546nm, expressed by uppercase letters) and blue light (495nm, expressed by lowercase letters) after RB treatment. **Z-2 Z-5 Z-8 Z-12** (×7), mean the fluorescence of seedling treated with RB germinated for 2d, 5d, 8d, 12d, respectively and their corresponding controls were showed by **Z-CK-2, Z-CK-5, Z-CK-8, Z-CK-12** (×7), respectively. **Y-6** (×10)**, Y-8** (×15)**, Y-12** (×20), mean the fluorescence of seedling cotyledon germinated for 6d, 8d, 12d, respectively and their corresponding controls were showed by **Y-CK-6** (×10)**, Y-CK-8** (×15)**, Y-CK-12** (×20)**,** respectively. **J-6** (×7)**, J-8** (×7)**, J-12** (×7), mean the fluorescence of seedling stem germinated for 6d, 8d, 12d, respectively and their corresponding controls were showed by **J-CK-6** (×7)**, J-CK-8** (×7)**, J-CK-12** (×7)**,** respectively. **J-H-8** (×10)**, J-H-12** (×15), mean the fluorescence of longitudinal sections of seedling stem germinated for 8d, 12d, respectively and their control was showed by **J-H-CK-8** (×15). **G-8** (×10)**, G-12** (×10), mean the fluorescence of seedling root germinated for 8d, 12d, respectively and their control was showed by **G-CK-8** (×10). The uppercase letters and their corresponding lowercase letters mean the fluorescence of the same place of seedling under different lights.

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