Australian Journal of Crop Science AJCS
AJCS 7(2):234-240 (2013) ISSN:1835-2707

A new effective fluorescent labeling method for anti-counterfeiting of tobacco seed using Rhodamine B

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Abstract

The present research was conducted to show a new anti-counterfeiting method using Rhodamine B (RB) as a fluorescent labeling on tobacco seeds. Seeds of three tobacco varieties, MS Yunyan85 (MS YY85), MS K326 and Honghua Dajinyuan (HHDJY) were soaked in RB solutions with different concentrations. The endosperm and embryo of treated seed showed bright red fluorescence under green light excitation (530-560 nm) under fluorescent microscope while no red color was observed in the control seeds. The red fluorescence was also observed in the leaf vein of 5- and 7-day-old treated seedlings. So, the bright red fluorescence can be used as an anti-counterfeiting marker for distinguishing true (treated) seed from fake (untreated) seeds. In addition, seed germination and seedling growth status were investigated after seed soaking. Through principal component analysis, the appropriate fluorescent labeling concentrations with no toxic effect to seed germination and seedling growth were obtained for each tobacco variety as: 0.1~0.5 mg ml^{-1} for MS YY85, 0.1 mg ml^{-1} for HHDJY and MS K326. Practically, optimal RB concentration should experimentally be determined for different crops and varieties as seed label. The regression equations of the relation between new principal component and RB concentration were established by the orthogonal polynomials regression analysis based on the combination of qualitative and quantitative traits, which could be used to predict the effects of different RB concentrations on germination of tobacco seeds. Therefore, soaking seed in suitable concentration of RB solution might be an effective fluorescent labeling method for anti-counterfeiting in tobacco seeds.

Keywords: Anti-counterfeiting, Fluorescent labeling, Rhodamine B, Tobacco seed, Seed germination.
Abbreviations: RB, Rhodamine B. GP, germination percentage. GI, germination index. VI, vigor index. RL, root length. SH, shoot height. DW, dry weight.

Introduction

The fake or counterfeit seeds that pretend to be high quality will seriously imperil the crop yield and quality in agriculture production (Gao and Zhou, 2005). Unfortunately, fake seeds still could be found in seed market (Wu et al., 2007). Therefore, anti-counterfeiting technologies on seed are extremely necessary to protect the seeds from being faked. Nowadays, for most seed corporations, packet tagging is widely used to protect the brand of seed companies (Zhang et al., 2007a; Gao and Zhou, 2005). However, these kinds of external anti-counterfeiting technologies would easily be imitated (Wang, 2009; Cai, 2009). In addition, seed is a special kind of business product. Sometimes we could not easily detect the authenticity of seeds by the naked eyes, especially after opening of package (Wu et al., 2007). Hence, fluorescent materials are used as anti-counterfeiting labels. They are directly applied on seed for anti-counterfeiting and source identification. When high quality seeds were treated with fluorescent dyes, they easily could be checked to find whether they are true or fake (or false) seeds.

Nowadays, some small fluorescent molecules such as fluorescein and rhodamine have become valuable tools in biology, and are ubiquitous as biomolecular labels, enzyme substrates, environmental indicators, and cellular stains (Waggoner and Kenneth, 1995; Johnson, 1998; Valeur, 2001; Goddard and Reymond, 2004; Sadaghiani et al., 2007). Moreover, paths of nutriment absorption and transmission in plant are often tracked through fluorescent markers. Shi et al. (2001) used fluorescent dyes to successfully mimic the performance of water moving into the wheat kernels. Compared with fluorescein, some key characteristics of rhodamines including low pH sensitivity, tunable spectral properties, better light stability and higher fluorescence quantum yields (Lavis and Raines, 2008) make them extensively used in biotechnology applications such as fluorescence microscopy, flow cytometry, fluorescence correlation spectroscopy and enzyme-linked immunosorbent assay (ELISA) (Liu, 2006). The simpler member of this class, rhodamine B (RB), which has better water solubility up to 50 g L^{-1} (Bedmar and Araguas 2002), making it easier to be applied to seeds through soaking. These properties laid the foundation for the application of RB on seeds as a label for anti-counterfeiting.

Many physiological parameters can be determined during screening of appropriate RB treatment and concentration. However, understanding of obtained data may some statistical data analysis methods. The principal component analysis (PCA) is the simplest of the true eigenvector-based multivariate

Table 1. Effect of Rhodamine B solutions on germination and seedling growth of tobacco seeds.

Variety	Rhodamine B concentration (mg ml^{-1})	GP (%)	GI	VI	RL (mm)	SH (mm)	DW (mg 100plants-1)
MS YY85	Ck	97.6a*	24.70a	0.274a	6.49c*	4.76ab	11.10a
	0.1	94.8a	24.12a	0.252ab	6.71c	4.59b	10.43bc
	0.3	96.4a	23.98a	0.252ab	8.28b	4.70ab	10.51b
	0.5	93.6a	23.56a	0.244b	10.13a	5.18a	10.35c
HHDJY	Ck	97.6a	24.46a	0.255a	7.41a	4.64a	10.42a
	0.1	98.0a	24.44a	0.260a	7.74a	4.59ab	10.64a
	0.3	97.6a	24.811a	0.237b	6.69b	3.75c	9.55c
	0.5	97.6a	24.68a	0.245b	6.11c	4.28b	9.92b
MS K326	Ck	98.8a	24.64a	0.245c	5.77c	4.49a	9.96c
	0.1	98.4a	24.50a	0.280a	6.69ab	4.34a	11.44a
	0.3	98.4a	24.20ab	0.264b	6.40b	3.83b	10.89b
	0.5	98.0a	23.68b	0.168d	6.97a	2.95c	7.114d

*Significant difference (α=0.05, LSD) among treatments within the same variety; Ck indicated the control treatment with water; GP=germination percentage, GI=germination index, VI=vigor index, RL=root length, SH=shoot height, DW=dry weight.

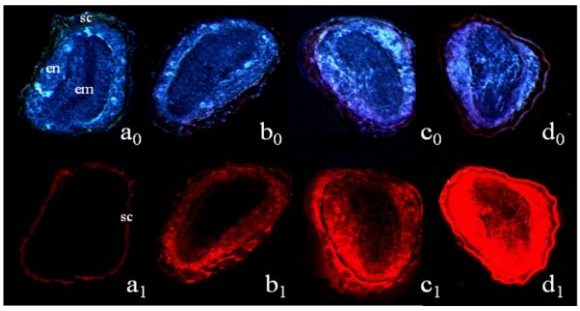

Fig 1. Fluorescent of MS YY85 seeds after soaking in Rhodamine B solutions for 24h under different concentrations illuminated different lights (the top line, excitation with natural light; the bottom line, excitation with green light (530-560 nm); **a_0**, the seed treated with distilled water under natural light; **a_1**, the seed treated with distilled water under green light excitation; **b_0**, the seed treated with 0.1 mg ml^{-1} Rhodamine B (RB) solution under natural light; **b_1**, the seed treated with 0.1 mg ml^{-1} RB solution under green light excitation; **c_0**, the seed treated with 0.3 mg ml^{-1} RB solution under natural light; **c_1**, the seed treated with 0.3 mg ml^{-1} RB solution under green light excitation; **d_0**, the seed treated with 0.5 mg ml^{-1} RB solution under natural light; **d_1**, the seed treated with 0.5 mg ml^{-1} RB solution under green light excitation; **em**, **en** and **sc** represent embryo, endosperm and seed coat, respectively.)

analyses (Yang et al., 2007). It involves a mathematical procedure that transforms a number of possibly correlated variables into a smaller number of uncorrelated variables called principal components. The first principal component accounts for much of the variability in the data as possible. However, only regression analysis can predict the effect and estimate of dynamic change and influence of the independent variable on dependent variable (Tang et al., 1997). The independent variables used in regression analysis are usually quantitative variables. Sometimes, qualitative variable such as crop variety if transformed to indicator variable (namely 0, 1 or -1) can also be used in regression analysis (Zhang et al., 2005; Cao et al., 2001).

In the present study, the effect of fluorescent labeling by RB for anti-counterfeiting was investigated on tobacco seeds. The dynamic changes of the influence of RB on seed vigor were also evaluated.

Results

Effects of soaking in RB solutions on seed germination and seedling growth

There was no significant differences in germination percentage (GP) and germination index (GI) among different treatments and varieties, except that 0.5 mg ml^{-1} RB significantly reduced the GI of MS K326 (Table 1). Treatments with 0.1 and 0.3 mg ml^{-1} had no significant effect on vigor index (VI) when compared with the control in MS YY85. For HHDJY, there were no significant differences between 0.1 mg ml^{-1} and control. However, application of 0.3 mg ml^{-1} RB significantly reduced the VI. For MS K326 variety, vigor index (VI) was significantly increased by seed soaking in 0.1 and 0.3 mg ml^{-1} solutions. VI of the three species was all significantly reduced under 0.5 mg ml^{-1}.

There were obviously differences in root length (RL), shoot height (SH) and dry weight (DW) of the studied varieties (Table 1). All soaking treatments increased root length of MS YY85, while the RL increase under 0.3 and 0.5 mg ml^{-1} of RB reached a significant level compared to control. The soaking treatments had no obvious influence on SH but decreased DW significantly. For HHDJY, 0.1 mg ml^{-1} treatment had no significant influence on seedling growth; however, the other two concentrations restrained RL, SH and DW significantly. All three treatments significantly enhanced RL of MS K326 compared with the control. In addition, 0.1 and 0.3 mg ml^{-1} treatments significantly increased DW, whereas the DW under 0.5 mg ml^{-1} treatment was significantly lower than the control.

Principal component analysis based on root length, shoot height and vigor index for each tobacco variety

The RL, SH and VI were used for principal components analysis of each variety because no significant differences existed in GP and GI among treatments and VI was the comprehensive representative of GI and DW.

Table 2. Analysis on the vector (Y_4) value of first principal component variable based on shoot height, root length and vigor index for each tobacco variety.

Variety	Rhodamine B concentration (mg ml^{-1})	Y_4*
MS YY85	Ck	3.192c**
	0.1	3.352c
	0.3	4.454b
	0.5	6.022a
HHDJY	Ck	10.624a
	0.1	10.856a
	0.3	9.454b
	0.5	9.551b
MS K326	Ck	3.482a
	0.1	3.419a
	0.3	2.998b
	0.5	0.779c

*The new principal component vector was used as a new integrated value. **Significant difference (α=0.05, LSD) among treatments within the same variety. Ck indicated the control treatment with water.

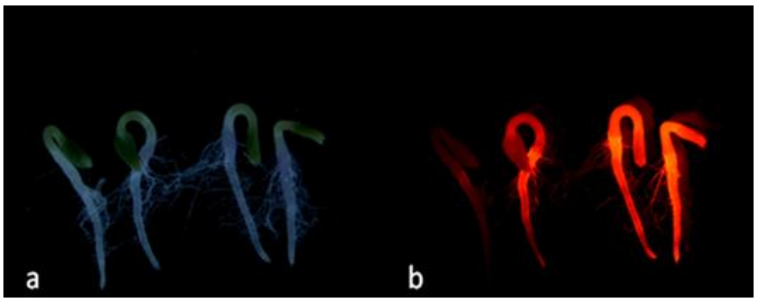

Fig 2. Seedling fluorescent of MS YY85 after germination for 5d under illumination of different lights (7×) (the four seedlings on each map, respectively, treated with distilled water (the control), 0.1, 0.3 and 0.5 mg ml^{-1} Rhodamine B solution from left to right. **a.** excitation with natural light, the treated seedlings showed no difference in seedling fluorescence from the control; **b.** excitation with green light (530-560 nm), treated seedling showed bright red fluorescence in whole seedling).

Due to the proportion of the first principal component, accumulative value of each variety reached 71.44%, 78.70% and 75.27 %, respectively. Therefore, the first principal component formula was used to calculate new principal component vector (Y_4) as a new integrated value.

The Y_4 of 0.3 and 0.5 mg ml^{-1} treatments were significantly improved, compared to control in MS YY85, in which the value increased with the increase of concentration (Table 2). For HHDJY, there was no significant difference in Y_4 between 0.1 mg ml^{-1} treatment and the control. The other two soaking treatments significantly reduced Y_4. .M K326 had similar results to HHDJY. However, the more obvious decrease was recorded in 0.5 mg ml^{-1} concentration in MS K326. In HHDJY, no significant difference was observed between the two soaking treatments.

Effects of RB treatments on Y_4 and seed vigor index based on qualitative combining with quantitative traits

Using combination of qualitative and quantitative traits, the regression relations between VI and RB concentrations and Y_4 and RB concentrations were predicted by orthogonal polynomial regression analysis.

Y_4 had linear relation with RB concentration, and VI had linear and quadratic relation with RB concentration. Both Y_4 and VI also had regression relation with the interaction between linear, quadratic and variety. All regression parameters are shown in the parentheses of Table 6. They reached highly significant levels, indicating that the two regression equations could effectively predict the relation among Y_4, VI, variety, RB concentration and their interactions. Then, the predicted values of Y_4 and VI were calculated according to their respective equations. The results showed that the difference between observed values and predicted values are very small (Table 7).

Effects of RB treatment on seed and seedling fluorescence

Under natural light, the control and all the treated seeds of MS YY85 did not have red fluorescence and showed no distinct difference (Fig 1: a_0, b_0, c_0, d_0). However, under illumination of green light, with excitation wavelength range of 530-560 nm, the inside of RB soaked seeds showed visible red fluorescence. When the soaking concentration increased, the red fluorescent intensity enhanced (Fig 1: b_1, c_1, d_1). Seed endosperm and embryo had brightest red fluorescence when seed treated with 0.5 mg ml^{-1} RB solution (Fig 1: d_1). However, the seed treated with distilled water only showed weak red fluorescence on seed coat under green light (Fig 1: a_1). This kind of red fluorescent was also detected in 5-day-old seedlings (Fig 2b). After seedlings grew for another 2d, the red fluorescence mainly existed in vascular bundle of shoot and cotyledon (Fig 3b), especially in leaf vein (Fig 3c). However, the control seedling showed no bright red fluorescence under green light. As excitation by natural light, there were no difference in seedlings color among treatments and the control (Fig 2a and 3a). The other two varieties had the similar results (data not shown).

Table 3. The transferring of qualitative trait (variety, A) to quantitative trait (X_1 and X_2).

Contrast ($df = 2$)	A_1* (MS YY85)	A_2 (HHDJY)	A_3 (MS K326)
X_1	1	0	-1
X_2	0	1	-1

* $\sum A_i = 0$, $i = 1, 2, 3$

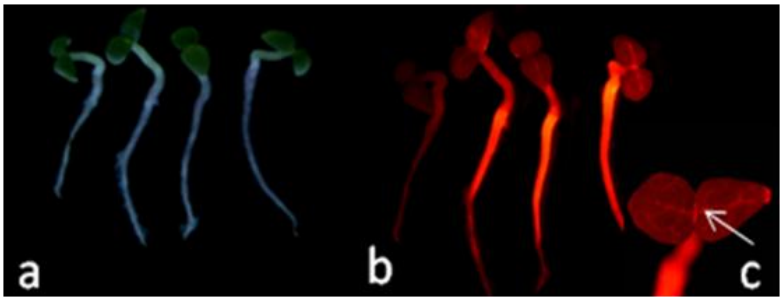

Fig 3. Seedling fluorescent of MS YY85 after germination for 7d under illumination of different lights (7×) (the four seedlings on each map respectively treated with distilled water (the control), 0.1, 0.3 and 0.5 mg·ml^{-1} Rhodamine B solution from left to right). **a.** excitation with natural light, the treated seedlings showed no difference in seedling fluorescence from the control; **b.** excitation with green light (530-560 nm), treated seedling showed bright red fluorescence in whole seedling, especially in vein; **c.** the 20 times magnifying seedling cotyledon treated with 0.1 mg ml^{-1} Rhodamine B solution, the vein showed bright red fluorescence (the arrow).)

Discussion

A chemical treatment used for seed anti-counterfeiting purpose must have no toxic effect on seed germination and seedling growth, which shows special color fluorescence under given irradiation light (Li et al., 2011). Then it could be used as an anti-counterfeiting marker for distinguishing true (treated) seed from fake (untreated) seed (Guan et al., 2011). In practice, the phytotoxicity of most fluorescent materials on seed germination and plant system is the major problem that limits their applications (Nair et al., 2011). However, there are still a few non- or less-toxic fluorescent compounds, such as fluorescein, which is used as a rapid sensitive and nonlethal method to detect skin ulceration in fish (Noga and Udomkusonsri, 2002). Fluorescein can improve the performance of plant growth and development, when applied in lower concentration. However, the growth of plants will be inhibited when applied in higher concentrations (Sellei et al., 1942; Li et al., 2011). Therefore, appropriate concentration of fluorescent compounds should be selected to enlarge its application. So, this experiment studied the effect of different soaking concentrations of RB, as new seed labeling method, on seed germination and seedling growth, by keeping track of the dye distribution and fluorescence in seed and seedling.

Through principal components analysis, the relatively appropriate soaking concentration for each tobacco variety was determined as following: 0.1~0.5 mg ml^{-1} for MS YY85, 0.1 mg ml^{-1} for HHDJY and MS K326, respectively. The variation observed in response to different RB concentrations in the three tobacco varieties might be due to the differences of chemical component and structure of seed coat or from the embryo sensitivity to RB, which was lowest in MS YY85 but comparatively higher in HHDJY and MS K326. So, in practical application, the RB concentration with no toxic effects on seed germination and seedling growth should be experimentally optimized. The regression equation could be used to effectively predict the influence of RB concentration variation on seed vigor of different tobacco varieties. If the RB concentrations did not optimized during the experiment, such as 0.15 or 0.25 mg ml^{-1}, the levels of tobacco seed vigor could also be predicted through regression equation. The same methods had been successfully developed by Zhang et al. (2005) to predict the effects of different Pb concentrations on rice seed vigor, and found that the degree of the restraint to plant growth would aggravate with the increasing of Pb concentration.

Seed inside showed bright red fluorescent under green light excitation, when seed soaked in RB solutions. As the solution concentration was increased, the fluorescent intensity in seed endosperm and embryo gradually intensified. It indicated that the fluorescent material can be transported through the tobacco seed coat and more amount of RB can be entered into the seed inside under higher the RB concentrations. In addition, this kind of red fluorescence was just be detected on seed coat of the control under green light. It might be caused by some component of seed coat which is not clear yet.

Seedlings treated with RB, especially the root and leaf vein, also showed bright red fluorescence under green light, compared with the control. This phenomenon in seedling would last for a period similar to the results reported in our previous study using Safranine T (Guan et al., 2011). It suggested that the fluorescent tracer might be up-taken by radical and then move up to the above-ground portion of seedlings after seed germinated at early stage. This speculation is consistent with the suggestion of Salanenka and Taylor (2006), studying the seed coat permeability and uptake of applied systemic compounds. Moreover, treated seedlings had no difference with the control under natural light. It meant that the treated seedling and the control could not be distinguished with the naked eyes under normal light. Only trough a special approach, such as the green light excitation, the special security label could be recognized, providing the confidentiality for RB as an anti-counterfeiting marker.

Different fluorescent dyes have different fluorescent characteristics (Lavis and Raines, 2008). When selecting efficient fluorescent dyes and labeling methods, the performance of fluorescent materials, the seed absorption characteristics and seed processing techniques should been taken into account (Salanenka and Taylor, 2006; Guan et al.,

Table 4. Establishment of orthogonal polynomials coefficient for quantitative trait (variety, A) within three levels.

Level	*k*=3	
	P_1 (Linear)	P_2 (Quadratic)
1	-1	1
2	0	-2
3	1	1
C	2	6
λ	1	3

k, Number of levels; λ, Constants; P_j, Contrast vector for *j*th degree orthogonal polynomial; *C*, Squared length for P_j.

Table 5. Factors and levels of orthogonal polynomials for quantitative trait (Rhodamine B concentration, B)

Contrast（*df*=2）	B_1	B_2	B_3	$\sqrt{\sum C_i^2}$
X_3（Linear）	$-1/\sqrt{2}$	0	$1/\sqrt{2}$	2
X_4 (Quadratic)	$1/\sqrt{6}$	$-2/\sqrt{6}$	$1/\sqrt{6}$	6

2011). The fluorescent dyes with no toxic effects on seed germination and growth which are visible only under certain wavelength of lights can be suggested as anti-counterfeiting labeling. Therefore, we suggest RB as a new anti-counterfeiting agent. This technology is difficult to be imitated suggesting a prospective utility in seed industry.

Materials and methods

Plant materials

Tobacco (*Nicotiana tabacum* L.) seeds (harvested at 2010) of three varieties, MS Yunyan85 (MS YY85), MS K326 and Honghua Dajinyuan (HHDJY), were obtained from Yunnan Provincial Academy of Tobacco Agricultural Sciences, China. There was no pedigree relationship among these selected tobacco varieties, which belong to flue-cured tobacco with a wide planting area in China. In addition, the earlier experiment proved they had different chilling tolerance (Li et al., 2009). Rhodamine B was obtained from Advanced Technology and Industrial Co., Ltd.

Fluorescent labeling by seed soaking

Tobacco seeds were soaked in RB solutions with 0.1, 0.3 and 0.5 mg ml^{-1} concentrations, respectively at 25°C in darkness for 24 h, and then dried back to their original moisture contents at room temperature. The seeds soaked in distilled water were used as the control (Ck).

Germination test

Germination test of treated seeds was conducted with three replicates of 100 seeds each. One hundred seeds were placed in a 9 cm diameter Petri dish with 3 layers blotter wetted by distilled water. All seeds were incubated in a germination chamber under alternating cycle of 8 h illumination at 30°C and 16 h darkness at 20°C for 16 days (ISTA, 2004). The germinated seeds (visible radical protrusion) were recorded daily for 16 days. Then, the germination percentage was calculated on the 16th day (ISTA, 2004). Whilst, the germination index (GI = ∑(Gt/Tt)) and vigor index (VI= GI×S) were calculated following Zhang et al. (2007b); Muharrem et al. (2008), where, Gt is the number of germinated seeds on days, Tt is time corresponding to Gt in days and S is the dry weight of 100 seedlings.

Root length and shoot height were measured manually with a ruler on twenty randomly selected seedlings and dry weights were determined after seedlings drying at 80ºC for 24 h.

Detection of fluorescence showing in seed and seedling

After the seeds were soaked in RB solutions with different concentrations in darkness for 24h, and seeds germinated for 5 and 7 days, fluorescence existed in seed and seedling was observed under fluorescence microscope (Leica MZ16FA) and photos were taken (Leica DFC42).

Statistical analysis

All data were analyzed by means of analysis of variance (ANOVA) using Statistical Analysis System (SAS) software. Percentage data were arcsin-transformed before analysis according to $\hat{y}$ = arcsin [sqr (x/100)]. Significant level α= 0.05 was used (Hu et al., 2005). Principal component analyses (Zhang et al., 2005) were conducted based on parameters of root length, shoot height and vigor index. Then the predicted regression formulas of the relation between seed vigor index and RB concentration, new principal component vector (Y) and RB concentration for each tobacco variety were constructed by the orthogonal polynomials regression analysis based on the combination of qualitative traits (variety) and quantitative traits (RB concentration): following by the predicted regression analysis in detail:

Tobacco variety was qualitative trait, so two variables X_1 and X_2 could be achieved by transferring the tobacco variety to indicator variable (Table 3). The RB concentrations in this experiment had three levels and each with an interval of 0.2 mg ml^{-1}. So, it was used as quantitative trait for orthogonal polynomials regression analysis (Jeffwu and Hamada, 2000; Mason et al., 2003). According to orthogonal polynomial coefficient for quantitative trait within three levels (Table 4), the factors and levels of orthogonal polynomials were established (Table 5).

The whole regression model was achieved according to the above conditions and was indicated as follows: $Y=b_0+b_1X_1+b_2X_2+\ldots+b_vX_v+\varepsilon$, where b_0, b_1, b_2,…, b_v is the parameters of regression model; X_1, X_2,…, X_v is the independent variables of regression model; ε is residual effect. Stepwise regression method was used to analyze Y_4 (new principal component vector) according to the whole regression model. Meanwhile, for the sake of validating the representation

Table 6. Predicting equations of regression model for Y_4, vigor index and Rhodamine B concentration.

Parameter	Predicting equation of regression model*
Y_4	$\hat{Y}_1$ =5.65395-1.10538X_1-2.14999X_2-0.30058X_3-1.87732X_1X_3 +0.31107X_2X_3-0.46206X_1X_4-0.29478X_2X_4 (b_0<0.0001,b_1<0.0001,b_2<0.0001,b_3<0.0001,b_{13}<0.0001,b_{23}<0.0001, b_{14}<0.0001, b_{24}<0.0001) **
Vigor index	$\hat{Y}_2$ =61.38741-1.82269X_1-8.07790X_3-1.85084X_4-8.99830X_1X_3 -2.69545X_2X_3-3.65625X_1X_4-2.49309X_2X_4 (b_0<0.0001,b_1<0.0001,b_3<0.0001,b_4=0.0005,b_{13}<0.0001,b_{23}<0.0001, b_{14}<0.0001, b_{24}<0.0001)

* $X_{(3)} = c_{(1)}/\sqrt{c_{(1)}} = \lambda_1\left(\frac{x-m}{\Delta}\right)/\sqrt{c_{(1)}}$, $X_{(4)} = c_{(2)}/\sqrt{c_{(2)}} = \lambda_2\left[\left(\frac{x-m}{\Delta}\right)^2 - \left(\frac{k^2-1}{2}\right)\right]/\sqrt{c_{(2)}}$;

** The value in parentheses is *P* value of each regression parameter

Table 7. Comparison of Y_4 and vigor index between real and predicted values by regression model.

Variety	Rhodamine B concentration (mg ml-1)	Y_4*	$\hat{Y}_4$	$(Y_4 - \hat{Y}_4)/Y_4$	*VI*	$\hat{VI}$	$(VI - \hat{VI})/VI$
MS YY85	0.1	3.352	3.343	0.0027	0.252	0.257	-0.0198
	0.3	4.454	4.472	-0.0040	0.252	0.255	-0.0119
	0.5	6.022	6.013	0.0015	0.244	0.247	-0.0123
HHDJY	0.1	10.856	10.847	0.0008	0.26	0.258	0.0077
	0.3	9.454	9.473	-0.0020	0.237	0.235	0.0084
	0.5	9.551	9.542	0.0009	0.245	0.243	0.0082
MS K326	0.1	3.419	3.410	0.0026	0.280	0.281	-0.0036
	0.3	2.998	3.017	-0.0063	0.264	0.264	0.0000
	0.5	0.779	0.770	0.0115	0.168	0.169	-0.0060

*Y_4 and *VI* were observed values; $\hat{Y}_4$ and $\hat{VI}$ were predicted values; $(Y_4 - \hat{Y}_4)/Y_4$ and $(VI - \hat{VI})/VI$ indicated the relative difference between Y_4 and $\hat{Y}_4$, and between *VI* and $\hat{VI}$.

of Y_4 as a new integrated parameter, regression predictive analysis was also used between VI of each tobacco variety and RB concentrations. The Y_4 ($\hat{Y}_1$) and VI ($\hat{Y}_2$) were used as dependent variables for linear regression analysis with repeated measurement data. The equations of regression prediction model were obtained as shown in Table 6.

Acknowledgements

The research was supported by key project of Natural Science Foundation of Zhejiang Province (No. Z3100150), the project of Yunnan Province Tobacco Company (No. 09YN008), Special Fund for Agro-scientific Research in the Public Interest (No. 201203052) and Major Special Project of Ministry of Agriculture (No. 2011ZX08005-005), P. R. China.

References

Bedmar AP, Araguas L (2002) Detection and the prevention of leaks from dams. Taylor & Francis, Lisse

Cai YL (2009) Several new anti-counterfeiting technologies for packaging. Shanghai Packaging 2: 40-41

Cao SQ, Lu W, Zhai HQ, Sheng SL, Gong HB, Yang TN, Zhang LX (2001) Research on the method to estimate flag leaf photosynthesis function duration at rice seedling stage by relative steady phase of chlorophyll content. J China Rice Sci 15: 309-313

Gao H, Zhou R (2005) The development trend of seed anti-counterfeiting packaging. Seed World 9: 6-7

Goddard JP, Reymond JL (2004) Enzyme assays for high-throughput screening. Curr Opin Biotechnol 15: 314-322

Guan YJ, Hu J, Li YP, Zheng YY (2011) A new anti-counterfeiting method: fluorescent labeling by safranine T in tobacco seed. Acta Physiol Plant 33: 1271-1276

Hu J, Zhu ZY, Song WJ, Wang JC, Hu WM (2005) Effects of sand priming on germination and field performance in direct-sown rice (*Oryza sativa* L.). Seed Sci Technol 33: 243-248

ISTA (2004) International ruled for seed testing 2004, ISTA Secretariat

Jeffwu CF, Hamada M (2000) Experiments: planning, analysis, and parameter design optimization. Wiley-Interscience, New York

Johnson I (1998) Fluorescent probes for living cells. Histochem J 30: 123-140

Lavis LD, Raines RT (2008) Bright Ideas for Chemical Biology. ACS Chem Biol 3: 142-155

Li YP, Guan YJ, Ma WG, Zheng YY, Hu J (2011) Effect of fluorescein on seed germination and seedling growth in tobacco. Acta Agri Univ Zhejiangensis 23: 31-34

Li YP, Xu SC, Ma WG, Zheng YY, Hu J (2009) Identification of chilling-tolerance in tobacco cultivars at germination and seedling growth stages. Bulletin Sci Tech 6: 806-810

Liu GH (2006) Fluorescence probe based on derivatives of fluorescein and rhodamine and its application. J Changzhi Univ 23: 17-21

Mason RL, Gunst RF, Hess JL (2003) Statistical design and analysis of experiments, with applications to engineering and science. 2nd edn. Wiley-Interscience, New York

Muharrem K, Gamze K, Mehmet DK, Mehmet A, Sevil S, Khalid MK, Cemalettin YC (2008) Interaction between seed size and NaCl on germination and early seedling growth of some Turkish cultivars of chickpea (*Cicer arietinum* L.). J Zhejiang Univ Sci B 9: 371-377

Nair R, Poulose AC, Nagaoka Y, Yoshida Y, Maekawa T, Kumar DS (2011) Uptake of FITC labeled silica nanoparticles and quantum dots by rice seedlings: effects on seed germination and their potential as biolabels for plants. J Fluoresc 21: 2057-2068

Noga EJ, Udomkusonsri P (2002) Fluorescein: a rapid, sensitive, nonlethal method for detecting skin ulceration in fish. Vet Pathol 39: 726-731

Sadaghiani AM, Verhelst SH, Bogyo M (2007) Tagging and detection strategies for activity-based proteomics. Curr Opin Chem Biol 11: 20-28

Salanenka YA, Taylor AG (2006) Seed coat permeability and uptake of applied systemic compounds. IV International

Symposium on Seed, Transplant and Stand Establishment of Horticultural Crops; Translating Seed and Seedling Physiology into Technology, San Antonio, Texas, 3-6 December 2006
Sellei J, Sellei H, Mayer A (1942) Effects of fluorescein on plant growth. Amer J Bot 29: 513-522
Shi YX, Hareland GA, Appolonia BLD (2001) The use of fluorescent dye to study the movement of water into wheat kernels. Zhengzhou Institute Tech 22: 17-20
Tang YH, Zhang SW, Gao RS, Wang CF (1997) The dynamic of rice quality during the ripening of rice kernel. Chinese J Rice Sci 11: 28-32
Valeur B (2001) Molecular Fluorescence: Principles and Applications. Wiley-VCH, Weinheim.
Waggoner A, Kenneth S (1995) Covalent labeling of proteins and nucleic acids with fluorophores. Methods Enzymol 246: 362-373
Wang ZH (2009) Anti-counterfeiting technologies grow by leaps and bounds in the market. Print Today 2: 76-79.
Wu P, Song CH, Zheng XY (2007) An advanced seed anti-counterfeiting technology and its application prospects. Vegetables, 11: 34-35.
Yang WQ, Hu J, Wu B, Zhang S, Song WJ (2007) Combining response surface analysis and principal component analysis to optimize the combination of GA_3 concentration in film coating and storage time of coated seed in cucumber. J Biomathematics 22: 323-328.
Zhang S, Hu J, Chen ZH, Chen JF, Zheng YY, Song WJ (2005) Effects of Pb Pollution on seed vigor of three rice cultivars. Rice Sci 12: 197-202.
Zhang S, Hu J, Zhang Y, Xie XJ, Allen K (2007b) Seed priming with brassinolide improves lucerne (*Medicago sativa* L.) seed germination and seedling growth in relation to physiological changes under salinity stress. Aust J Agr Res 58: 811-815
Zhang YM, Sun WY, Xu M (2007a) The application of anti-counterfeiting packaging to seed industry. Seed 11:16-17