JOURNAL OF INTEGRATED OMICS
A METHODOLOGICAL JOURNAL
HTTP://WWW.JIOMICS.COM

ORIGINAL ARTICLE | DOI: 10.5584/jiomics.v1i2.70

Proteomic approach for molecular physiological mechanism on consecutive monoculture problems of *Rehmannia glutinosa*

Wenxiong Lin [1,2*], Changxun Fang [2,#], Linkun Wu [2,#], Gailing Li [2,#], Zhongyi Zhang[1]

[1]Key Laboratory of Ministry of Education for Genetics, Breeding and Integrated Utilization of Crop (FAFU), Fuzhou 350002, Fujian, P. R. China; [2] School of Life Sciences, Fujian Agriculture and Forestry University, Fuzhou 350002, Fujian, P. R. China. # These authors contributed equally.

Received: 16 August 2011 **Accepted:** 13 December 2011 **Available Online**: 28 December 2011

ABSTRACT

Rehmannia glutinosa, a famous Chinese medicinal plant, is not suitable for consecutive monoculture, because there are autotoxic metabolites excreted by its tuberous roots, which can greatly limit the plant growth and development. In this research, cultivar "Wen 85-5" *R. glutinosa* under three different cultivation modes, including the newly planted, the two-year and three-year consecutively monocultured, was used in the field test. The differential expression of leaf proteins, physiological changes and corresponding medicinal quality of tuberous roots at the early tuberous root enlargement stage were detected and compared in different years of consecutive monoculture. The results showed that consecutive monoculture resulted in decrement of chlorophyll content, photosynthetic capacity and root activity, but increases in free radicals and lipid peroxidation. Furthermore, the content of catalpol, the main medicinal ingredient in *R. glutinosa* tuberous roots was analyzed by FTIR and HPLC. The result showed that consecutive monoculture resulted in declined medicinal ingredients. Comparative proteomics analysis revealed 20 differentially expressed protein spots in response to increasing years of monoculture. Among them, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) kinase, Rubisco, sedoheptulose-1,7-bisphosphatase related to Calvin cycle, and other proteins, i.e. proteasome, malonyl CoA-ACP transferase, antioxidases, pathogenesis-related protein and mRNA-binding protein were down-regulated with increasing years of monoculture. While energy metabolism related proteins (ATP synthase subunit β, ATPase, ATP-binding protein) and stress response related proteins (heat shock proteins) were up-regulated. Therefore it was concluded that consecutive monoculture of *R. glutinosa* remarkably affected the physiological reactions and induced the changes in the expression of leaf proteins, this in turn had a negative impact on the biomass and its quality of the medicinal plants.

Keywords: *R. glutinosa*; consecutive monoculture problem; proteomics; medicinal ingredient.

Abbreviations

AA-MA pathway acetate-malonate pathway; **ACP** Acyl carrier protein; **Ci** intercellular CO_2 concentration; **CoA** coenzyme A; **2-DE** two-dimensional electrophoresis; **FTIR** Fourier Transform Infrared Spectroscopy; **Gs** stomata conductance; **HPLC** High-performance liquid chromatography; **MDA** malondialdehyde; **MS** Mass Spectrometry; **NP** the newly planted; **PMF** Peptide Mass Fingerprinting; **Pn** net photosynthetic rate; **RA** reduction amount of TTC; **RFW** fresh weight of roots; **Rubisco** ribulose-1,5-bisphosphate carboxylase/oxygenase; **SM** the two-year consecutively monocultured; **TM** the three-year consecutively monocultured; **TTC** Triphenyl-Tetrazolium Chloride; **Tr** transpiration rate.

1. Introduction

Consecutive monoculture problems refer to that when growing one crop consecutively in the same field, unfavorable results would be brought about even though under normal cultivation management, which include retardation in plant growth, declines in crop yield, deterioration in growth conditions, and increases in pest damage, etc, [1]. The phenomenon is very common in the cultivation of Chinese medicinal plant, especially in *Rehmannia glutinosa* [1]. Accord-

***Corresponding author:** Wenxiong Lin, Agroecological Institute, Fujian Agriculture and Forestry University, Fuzhou, Fujian 350002, China. Tel: +86059183769440; Fax: +86059183769440; E-mail Address: lwx@fjau.edu.cn.

ing to statistics, about 70% medicinal plants suffer from various degrees of consecutive monoculture problems, which are adversely affecting plant yield and quality, limiting the sustainable utilization of Chinese medicinal resources, and further restricting the development of Chinese medicine industry. Previous researches suggested that there were three main reasons for plant consecutive monoculture problems, such as decline in soil fertility [2], serious pest damage caused by soil disease [3, 4], and autotoxicity of plant root exudates [5]. But many researchers believed that the latter is the resultant factors [6, 7, 8].

R. glutinosa, Scrophulariaceae perennial herb, is useful in many medicinal aspects, such as hemostat, diuretic, anti-inflammatory, anti-fungal, anti-radiation, and even in the treatment of liver disorders and diabetes. Thus it has been listed as one top-grade medicinal ingredient in China pharmacopoeias, and one of the highest consumed medicinal materials in China [9]. However, the consecutive monoculture problems in *R. glutinosa* are very prominent, which lead to retardation of plant growth and development, inhibition on enlargement of tuberous roots (the main medicinal part), thus decreases in yield and quality of medicinal plants. Furthermore, to overcome the obstacle, a necessary interval of 8 -10 years from first cropping of *R. glutinosa* to its next cropping in the same field would really affect the corresponding production and regional economy development of *R. glutinosa* to a great degree [10].

There are many kinds of allelochemicals with autotoxicity in the *R. glutinosa* rhizospheric soil for first cropping [11]. These allelochemicals can be converted by soil microorganisms, and result in changed soil microflora and the toxicity of substance that remains in soil [12]. Thus the growth of next cropping is greatly suppressed by these autotoxic exudates from standing crop roots. Therefore, further research on self-poisoning-associated molecular mechanism of consecutively monocultured *R. glutinosa* can provide a theoretical basis for the application of ecological cultivation techniques in the regulation of *R. glutinosa* development.

2. Material and methods

2.1 *Plant samples*

R. glutinosa "Wen 85-5", a cultivar produced largely in industry scale was used as crop material [13, 14]. In general, *R. glutinosa* was planted on April 15 and harvested on October 30. Three different kinds of soils from Jiaozuo city, Henan province, China (35°19'N, 113°51'E) were used for cultivation, including i) control soil with no *R. glutinosa* cultivation (No.1), ii) one-year cultivated soil (No.2), iii) two-year consecutively monocultured soil (No.3). The control plots were left fallow for the entire duration of the experiment (starting from April 15, 2006 to October 30, 2007). The one-year cultivated *R. glutinosa* plots were left fallow for the first year (2006) of the experiment, and then planted on April 15, 2007 and harvested on October 30, 2007. The two-year cultivated plots were planted on April 15, 2006, harvested on October 30, 2006, left fallow until April 14, 2007, replanted on April 15, 2007 and harvested on October 30, 2007. These three kinds of soils were collected at the same time (November 2, 2007). The physical and chemical properties of these three kinds of soils were shown in the Table 1. After dried at 70°C for 2 hours, the three different kinds of soils were pulverized and sieved (2mm mesh) respectively to remove rough granules and plant root residues, and then used separately for pot-cultivation of *R. glutinosa* in the greenhouse of Agroecological Institute, Fujian Agriculture and Forestry University on May 15, 2008. Each treatment had five replicates. The *R. glutinosa* plants grown in the corresponding three kinds of soils (No.1, No.2, No.3) were labeled respectively as the newly planted (NP), the two-year consecutively monocultured (SM) and the three-year consecutively monocultured (TM). Then we collected the plant samples from five replicates of each treatment at the early tuberous root enlargement stage and mixed together to make composite samples for further analysis.

2.2 *Determination of root activity of R. glutinosa*

Root activity could be reflected by the root dehydrogenase activity and measured by Triphenyl-Tetrazolium Chloride (TTC) method [15]. Optical density value in 485 nm (OD_{485}) of various samples were measured and then converted to corresponding reduction amount (*RA*) of TTC through related formula to indicate root activity (mg/g·h FW) = *RA*/

Table 1. Physical and chemical properties of soils from the control, one-year and two-year cultivated plots.

Soils	OM (g/kg)	TN (g/kg)	TP (g/kg)	TK (g/kg)	AN (mg/kg)	AP (mg/kg)	AK (mg/kg)	pH
No.1	10.04c	2.46b	0.51c	1.14b	21.63b	34.27c	305.76b	13.37a
No.2	10.35b	1.49c	0.68b	1.18a	18.40c	37.37b	294.00c	13.27b
No.3	11.17a	2.81a	0.85a	1.09c	24.33a	54.20a	312.53a	13.28b

Note: No.1, control soil; No.2, one-year cultivated soil; No.3, two-year monocultured soil. OM, organic matter; TN, total nitrogen; TP, total phosphorus; TK, total potassium; AN, available nitrogen; AP, available phosphorus; AK, available potassium. Column values followed by different letters were significantly different ($P \leq 0.05$, $n = 3$) determined by ANOVA followed by Tucky's tests.

RFW·T, where, *RFW*= fresh weight of roots, *T*= incubation time.

2.3 *Determination of main medicinal ingredients of R. glutinosa tuberous roots by Fourier Transform Infrared Spectroscopy (FTIR) and High-performance liquid chromatography (HPLC)*

The samples were sieved separately by 100 mesh sifter (aperture 140 μm) after ustulation and pulverization, then 1.5 mg of the sifted powders were placed in an agate mortar for rough grinding before adding 100 mg dried potassium bromide powders for further mixing and grinding. The mixture obtained above was put into the pressure machine for 1.5 min under 16 Mpa, then a piece of sample with 13 mm in diameter and 0.3 mm in thickness was got for Fourier Transform Infrared Spectroscopy analysis using AVATAR360-infrared spectrometer (Thermo Nicoler, USA).

The High-performance liquid chromatography setting parameters were as follows: chromatography column- 18 alkyl Silanes-bonding silica column, mobile phase- acetonitrile/0.1% phosphoric acid solution (volume ratio is 1/99), velocity- 1.0 mL/min, measuring wavelength- 210 nm, theoretical plates- 5000, column temperature- 25°C. Sample preparation was as follows: after drying under low pressure in 80°C for 24 hours, the sample was ground into coarse powder, into 0.4 g powders 25ml methanol was added before extracting by heating circumfluence for 1.5 h, then the liquid was filtrated, concentrated, and confected to 10 mL using mobile phase. The 10 ug/ml catalpol solution was used as standard control.

2.4 *Determination of chlorophyll content of R. glutinosa*

Three complete leaves per plant were collected for chlorophyll content determination by chlorophyll apparatus (SPAD-502, Konica Minolta, Japan) [16] at the early tuberous root enlargement stage of *R. glutinosa*. The determination was repeated three times, and statistic analysis of the results was carried out by DPS software (version 7.05) using one way analysis of variance (ANOVA) followed by Tucky's tests.

2.5 *Determination of photosynthesis ability of R. glutinosa*

In the meantime, net photosynthetic rate [Pn, μmol/(m^2·s)], transpiration rate [(Tr, mmol/(m^2·s)], stomata conductance [Gs, mmol/m^2·s)], and intercellular CO_2 concentration [Ci, μmol/mol] of the fourth leaf counted from center outward per plant were determined by portable photosynthesis apparatus (LI-6400, LI-COR, USA) and statistic analysis of the results was carried out by DPS software (version 7.05) using one way analysis of variance (ANOVA) followed by Tucky's tests.

2.6 *Determination of malondialdehyde (MDA) content of R. glutinosa leaves*

The detailed operation was conducted according to Esterbauer and Cheeseman [17]. Then OD_{532}, OD_{600}, and OD_{450} value were respectively measured and applied to the formula [C (μmol/L) = $6.45\cdot(OD_{532}-OD_{600})-0.56\cdot OD_{450}$], for further calculation of MDA content (nmol/g FW).

2.7 *Protein Separation*

After TCA/acetone extraction, the leaf proteins were separated through polyacrylamide gel with two-dimensional electrophoresis (2-DE) referring to O'Farrell's [18] and Wu *et al.*'s [19] methods. After electrophoresis, the gels were silver stained according to Vorum *et al*'s [20] method, scanned with Imagescan and analyzed by ImageMaster software 5.0 (GE Healthcare, Uppsala, Sweden) to choose the differentially expressed protein spots for next in-gel digestion with Bergman *et al*'s [21] method.

2.8 *Mass Spectrometry (MS) analysis*

The digested proteins were analyzed using an ABI 4700 Proteomics Analyzer (Applied Biosystems, Foster city, USA) mass spectrometer with TOF/TOF™ optics equipped with a 200-Hz frequency-tripled Nd: YAG laser operating at a wavelength of 355 nm. Mass spectra in the range of m/z 700 to 3500 were acquired in the positive ion mode. The five most intense ions per spot were selected for subsequent MS/MS analysis. Collision-induced dissociation was performed using a collision energy of 1 keV in a collision cell with a gas pressure of 6×10^{-7} Torr. External mass calibration was applied using the ABI 4700 calibration mixture (Applied Biosystems, Framingham, MA, USA).

2.9 *Database search*

Based on the combined MS+MS/MS data, peptide and protein identifications were performed by searching against NCBInr database (2009.09.15, 9694989 sequences) using GPS Explorer™ software 2.0 (Applied Biosystems, Foster city, USA) with MASCOT 2.2.03 search engine (Matrix Science Ltd, London, UK). The parameters were as follows: taxonomy was viridiplantae (Green Plants), trypsin digestion with one missed cleavage was selected, mass tolerance was 100 ppm for the precursors and 0.6 Da for the MS/MS ions, methyl methanethiosulfonate- labeled cysteines were set as fixed modifications, oxidized methionines were set as variable modifications. The self-degradation peak of trypsin and pollutants peak were removed manually before retrieval.

3. Results

3.1 *Root activity of R. glutinosa*

The root activity of two-year (SM) and three-year (TM) consecutively monocultured *R. glutinosa* was significantly reduced by 53.1% and 73.9% respectively compared to the newly planted (NP) (Figure 1), suggesting that normal physiological metabolism of roots was greatly inhibited with poor performance of root activity and decline in root dehydrogenase activity under consecutive monoculture.

3.2 *Analysis of main medicinal ingredients in R. glutinosa tuberous root*

The results from FTIR analysis (Figure 2) showed that the main medicinal ingredients in terms of the species of functional groups were not significantly different between NP and SM. From FTIR detection, it also could be found that the main medicinal ingredients in *R. glutinosa* with different monoculture years shared the same chemical compositions, suggesting that consecutive monoculture had no significant effect on the main medicinal ingredients of *R. glutinosa* (Table 2).

Compared HPLC profiles of tested samples (relative concentration was 16 mg/ml) with that of catalpol standard liquid (10 μg/mL), we found that peak 3 in the retention time of 6.4 min was the characteristic peak of catalpol (Figure 3). Then the relative content of catalpol in tuberous roots of newly planted (NP) and two-year consecutively monocultured (SM) *R. glutinosa* was calculated according to that of standard catalpol liquid, with 0.88% and 0.63% (w/w) respectively, both in accordance with the content requirement mentioned in Pharmacopeia (no less than 0.2%, w/w). There were five peaks in total shared both by NP and SM, among them peak 1 was regarded as solvent peak emerging between 2 and 4 min, peak 5 showed no significant changes, the area of peak 2 and peak 4 of SM were both greater than NP, while peak 3 showed the opposite result,

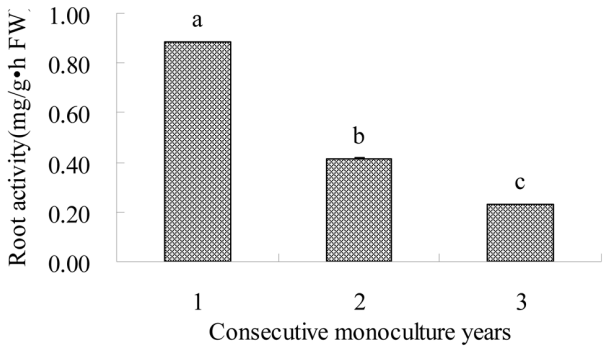

Figure 1. The effect of consecutive monoculture on root activity of *R. glutinosa*. The numbers 1, 2 and 3 on the abscissa represent the plants from the newly planted (NP), the two-year (SM) and three-year (TM) consecutively monocultured, respectively. Different letters show significant differences at the 5% level according to its p-value by Tucky's test (P≤0.05, n = 3).

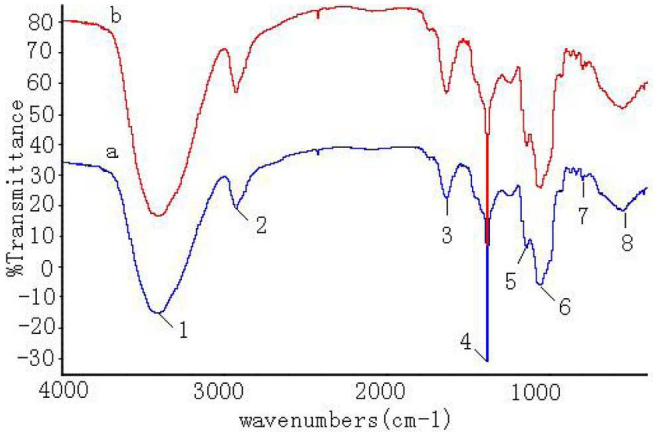

Figure 2. The FTIR analysis on the medicinal ingredients of newly planted (NP) and two-year monocultured (SM) *R. glutinosa* tuberous roots. a: newly planted *R. glutinosa*, b: two-year consecutively monocultured *R. glutinosa*.

Table 2. FTIR analysis of functional groups of medicinal ingredients in the tuberous roots of newly planted (NP) and two-year consecutively monocultured (SM) *R. glutinosa*

Peak Number [a]	Wavelength (λcm^{-1})		Functional group
	Newly planted (NP)	Consecutively monoculture (SM)	
1	3415.99	3407.65	—OH (association reaction)/ stretching vibration
2	2927.93	2927.93	methylenedi/ 2930、2850
3	1630.59	1643.11	C=C/ 1690～1600/ stretching vibration
4	1388.64	1388.64	methyl/ 1380
5	1142.53	1146.70	—S=O/ 1200～1040/ stretching vibration
6	1050.75	1059.10	C—O/ 1300～1000/ stretching vibration
7	796.29	796.29	C—Cl/ 800～600/ stretching vibration
8	554.35	554.35	C—Br/ 600～500/ stretching vibration

Note: [a] The number corresponds to the spectrum in Figure 2.

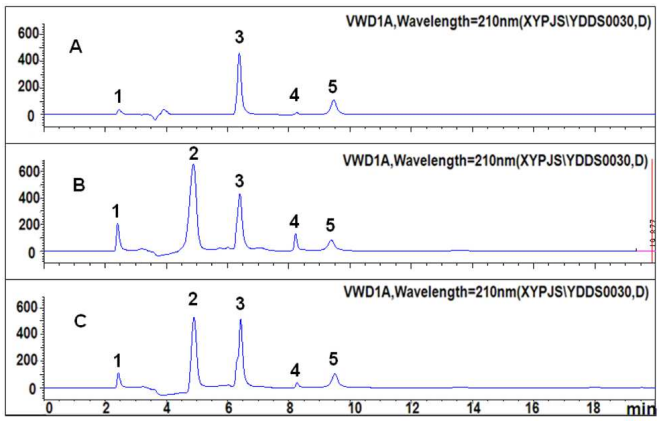

Figure 3. The HPLC analysis of the catalpol content of newly planted (NP) and two-year monocultured (SM) *R. glutinosa* tuberous roots. A: standard control, B: two-year consecutively monocultured *R. glutinosa,* C: newly planted *R. glutinosa*.

but no significant difference was shown in the sum of peak area 2, 3 and 4 between NP and SM. The result suggested that the effect of consecutive monoculture on medicinal quality of *R. glutinosa* was mainly reflected in the decline of content catalpol, a main medicinal ingredient in *R. glutinosa*.

3.3 *Differential expression of R. glutinosa leaf proteins under different cultivation modes*

Using 2-D electrophoresis, differential expression profiles of proteins extracted from three different kinds of *R glutinosa* leaves were established (Figure 4). Then imageMaster 5.0 software was applied to analyze the 2-D profiles to find out those proteins with differential expression (Figure 5). Finally, a total of 20 protein spots with *p*I in the range of 5- 8, MW about 14- 116 kDa, were differentially expressed, i.e., their intensities varied, at least on one gel in comparison to the control (NP), by more than 1.5-fold. Twelve spots (spots 6, 7, 9, 10, 13, 16, 22, 24, 25, 27, 29, and 31 constituting 60% of the total differentially expressed proteins) were down-regulated with the increasing years of monoculture. Five spots (spots 4, 14, 21, 33 and 35, constituting 25% of the total differentially expressed proteins) were up-regulated with the increasing years of monoculture. Three spots (spots 12, 30 and 32, constituting 15% of the total differentially expressed proteins) were down-regulated only in the three-year monoculture, but none in the two-year monoculture.

These 20 differentially expressed proteins were successfully identified by MS analysis (Table 3), and then divided into 5 classes according to their respective functions through bioinformatics searching (KEGG database, http://www.genome.jp/kegg/): (I) photosynthesis related proteins (spots 6, 7, 24 and 29, constituting 20%), (II) metabolism related proteins (spots 9, 10, 12, 13, 22, 25 and 32, constituting 35%), (III) energy conversion related proteins (spots 4, 21, 30 and 33, constituting 20%), (IV) resistance related proteins (spots 14, 16, 27 and 35, constituting 20%), (V) nucleotide metabolism related protein (spot 31, constituting 5%). The results showed that most proteins related to photosynthesis, mRNA binding (including the chloroplast stem-loop binding protein-41), protein degradation (including the proteasome) and pathogenesis were down-regulated with the increasing years of monoculture. However, the proteins involved in energy metabolism and stress/defense response (including the class-1 LMW heat shock protein and cytosolic class I small heat shock protein) were up-regulated with the extended monoculture.

3.4 *Chlorophyll content of R. glutinosa leaves*

The chlorophyll content of *R. glutinosa* leaves decreased with the increasing years of monoculture, showing that the

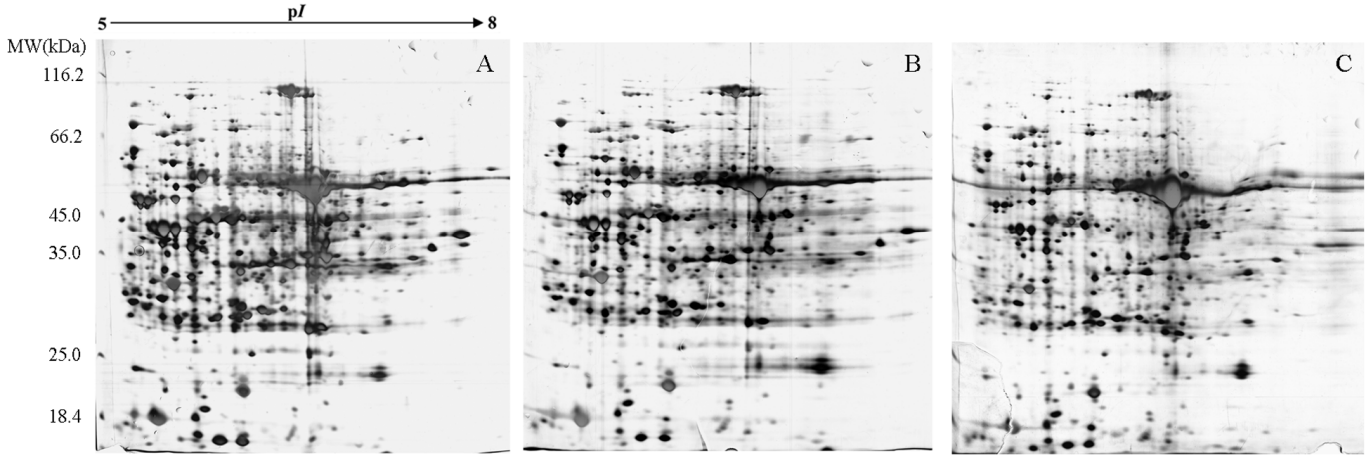

Figure 4. The silver stained 2-DE gel of proteins extracted from the leaves of newly planted (A), two-year (B) and three-year (C) consecutively monocultured *R. glutinosa*. In the first dimension, 160μg of protein was loaded on a 17 cm strip with a nonlinear gradient of pH 5–8. In the second dimension, a 5% stacking gel and a 10% separating gel were used. Unstained protein molecular weight markers ranging from 14.4 to 116.2 kDa (Promega, Madison, USA) were used in the second dimension for size standardization.

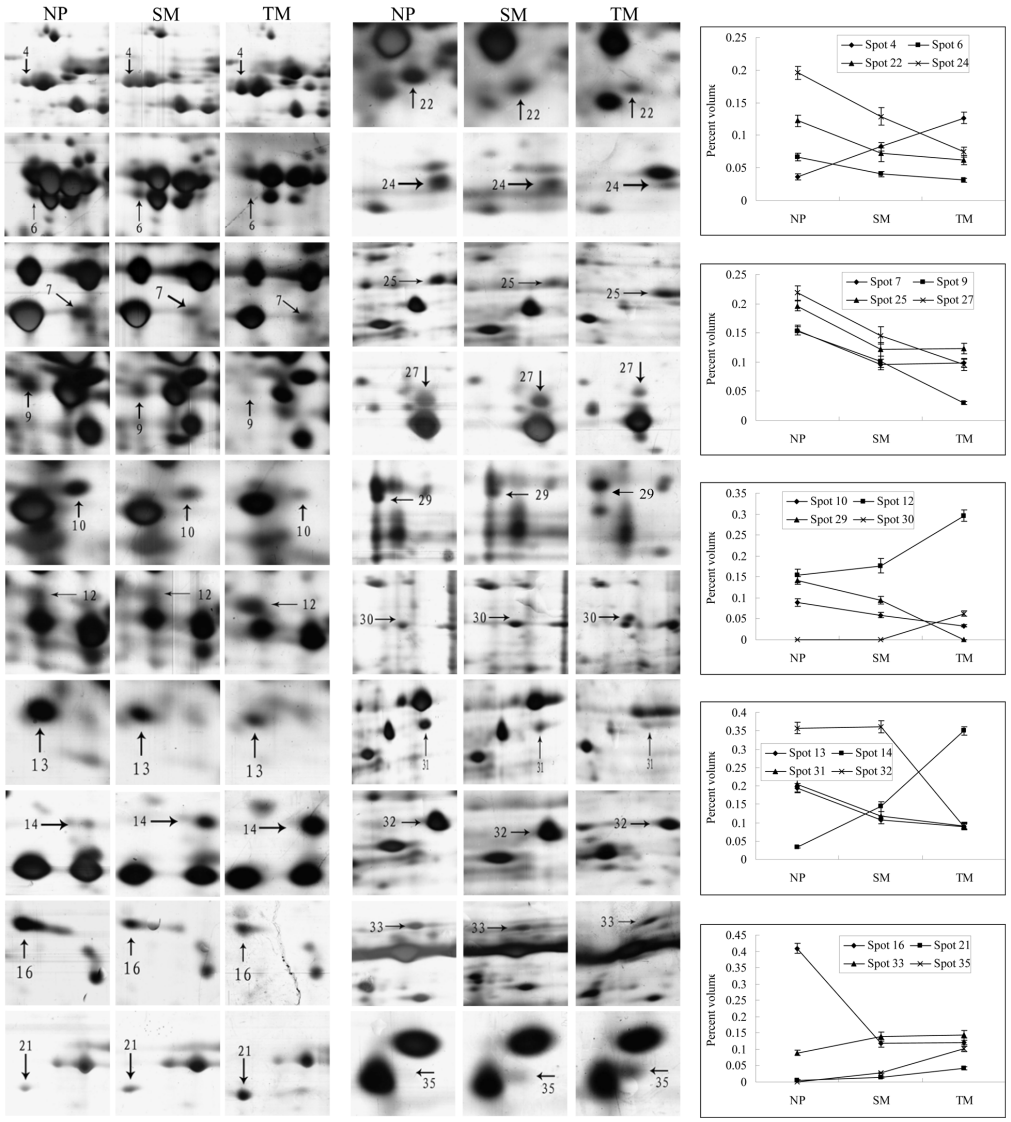

Figure 5. Close-up views of the differentially expressed proteins extracted from different *R. glutinosa* leaf samples in response to different-year consecutive monoculture (A) and relative abundance in terms of fold change of each spot in the gel (B). NP: proteins extracted from newly planted *R. glutinosa leaves*, SM: proteins extracted from two-year consecutively monocultured *R. glutinosa leaves*, TM: proteins extracted from three-year consecutively monocultured *R. glutinosa leaves*.

chlorophyll content of newly planted *R. glutinosa* leaves was significantly higher by 23.3% and 27.8% than those of two-year and three-year consecutively monocultured *R. glutinosa* leaves, respectively (Figure 6). The result suggested that consecutive monoculture caused the damage of photosynthesis system in *R. glutinosa* leaves, and had a great effect on the balance between chlorophyll synthesis and degradation, leading to a decline of photosynthesis capacity in plant (Figure 7).

3.5 *Photosynthesis-associated indicators of R. glutinosa*

As shown in Figure 7, photosynthetic rate (Pn), transpiration rate (Tr), stomata conductance (Gs) all significantly declined while intercellular CO_2 (Ci) increased with the increasing years of monocultue. It confirmed that photosynthetic capacity of *R. glutinosa* was greatly affected by consecutive monoculture.

3.6 *MDA content of R. glutinosa*

As one of membrane lipid peroxidation indicators, variation of MDA content would reflect the corresponding changes in membrane lipid peroxidation and plant growth. It was shown that the MDA content of two-year (SM) and three-year (TM) consecutively monocultured *R. glutinosa* were higher than that of the newly planted (NP) by 43.1% and 73.1%, respectively (Figure 8). It suggested that membrane lipid peroxidation could be greatly intensified by consecutive monoculture. Lipid, a major component of cell membrane, is correlative to the normal intracellular metabolism to some extent, thus its peroxidation will directly lead to

Table 3. The differentially expressed proteins identified by MALDI-TOF/TOF-MS in *R. glutinosa* leaves under different cultivation conditions

Spot no.	Accession number [a]	Protein name	Theoretical *PI*/ MW (kDa) [b]	PMF/ Peptides [c]	Score [d]	Score C. I. % [e]	Species [f]
6	gi|13430334	Ribulose-bisphosphate carboxylase (RuBisCO) activase	6.7/37.0258	6/3	229	100	*Zantedeschia aethiopica*
7	gi|68565781	RuBisCO activase 2	6.78/48.0231	11/3	134	100	*Zantedeschia aethiopica*
24	gi|3116024	Ribulose-bisphosphate carboxylase large subunit	6.2/51.8881	12/0	70	97.326	*Pycreus nuerensis*
29	gi|5817374	Ribulose 1,5-bisphosphate carboxylase large subunit	6.57/48.6817	9/2	126	100	*Angelonia pubescens*
9	gi|115457386	Sedoheptulose 1, 7-diphosphate	5.64/42.2181	10/2	79	99.671	*Oryza sativa*
10	gi|12003283	Malonyl-CoA:ACP transacylase	5.91/39.6506	3/2	71	98.15	*Perilla frutescens*
12	gi|73808462	Putative S-adenosylmethionine decarboxylase proenzyme	5.04/37.7408	11/0	63	88.055	*Solanum lycopersicoides*
13	gi|12229923	Proteasome subunit alpha type 5 (20S	4.7/25.9639	10/2	109	100	*Glycine max*
22	gi|46399269	Putative pyridoxine biosynthesis protein	5.93/33.068	18/2	181	100	*Nicotiana tabacum*
25	gi|82941449	Fructose-bisphosphate aldolase	6.47/38.1366	9/1	76	99.344	*Codonopsis lanceolata*
32	gi|1168408	Fructose-bisphosphate aldolase, cytoplasmic isozyme 1	6.38/38.4219	6/1	137	100	*Pisum sativum*
4	gi|15241847	ATP binding	5.03/71.3422	15/3	211	100	*Arabidopsis thaliana*
21	gi|18417676	ATP binding / ATPase/ nucleoside-triphosphatase/nucleotide binding / protein binding	5.93/108.8757	24/3	207	100	*Arabidopsis thaliana*
30	gi|115469766	UTP-glucose-1-phosphatetransferase	6.4/67.4665	6/3	70	97.617	*Oryza sativa*
33	gi|17224782	ATP synthase beta subunit	5.15/53.3608	18/1	148	100	*Stemona japonica*
14	gi|25044839	Class-1 LMW heat shock protein	6.77/17.5621	5/1	116	100	*Ananas comosus*
16	gi|38344034	OJ991214_12.15	4.67/18.1001	5/3	164	100	*Oryza sativa*
27	gi|510940	Pathogenesis related protein	7.19/16.4715	9/0	77	99.566	*Asparagus officinalis*
35	gi|37704433	Cytosolic class I small heat shock protein	5.8/14.0172	6/1	68	96.045	*Nicotiana tabacum*
31	gi|15229384	Chloroplast stem-loop binding protein-	8.54/43.9025	6/2	102	99.998	*Arabidopsis thaliana*

Note: [a] GI number in NCBI. [b] Theoretical p*I* and molecular weight. [c] The number of peptides identified by MS or MS/MS. [d] Protein scores were taken from the search results combined MS and MS/MS using GPS Explorer software (Version 2.0). In this program, a mascot score > 62 was considered significant ($p<0.05$). [e] The Confidence Interval (C.I. %) for the protein score. f) The scientific name of green plants corresponding to the best-matching proteins.

a disturbance of intracellular balance and various metabolisms, and then have a further severe effect on plant's normal development. The results above confirmed that the normal development of consecutively monocultured *R. glutinosa* was suppressed compared with that of the newly planted [13], especially with the increasing years of monoculture.

4. Discussion

Consecutive monoculture problems represent a major issue which greatly plagues the modern production of Chinese medicinal plant, thus the relevant research is very important for further elucidating its physiological mechanism at the molecular level. Our previous study found that vanillic acid, β-sitosterol and some other second metabolites excreted by *R. glutinosa* roots were the main substances with autoxicity and greatly limited the development of its next cropping [11]. In the present study, we found that the root activity of *R. glutinosa* decreased gradually with the increasing years of monoculture, which in turn intensified the damage degree on the whole plant growth. Furthermore, consecutive monoculture resulted in decrement of chlorophyll content, stomata conductance and photosynthesis rate, and increase in intercellular CO_2 concentration, which all together led to a decline of photosynthesis ability, whilst increment of MDA content and intensification of membrane lipid peroxidation in the leaves of consecutively monocultured *R. glutinosa* led to the deterioration of crops growth.

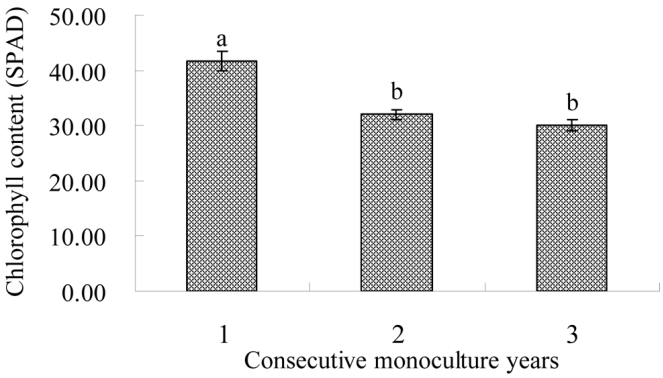

Figure 6. The effect of consecutive monoculture on chlorophyll content of *R. glutinosa.* The numbers 1, 2 and 3 on the abscissa represent the plants from the newly planted (NP), the two-year (SM) and three-year (TM) consecutively monocultured, respectively. Different letters show significant differences at the 5% level according to its p-value by Tucky's test (P≤0.05, n = 3).

The results from comparative proteomic analysis showed that mRNA-binding protein was down-regulated in the consecutively monocultured *R. glutinosa*. Combined with some proteins, mRNA can prolong its life span and stability in the whole process from mRNA transcription to degradation though the half-life of mRNA in cell is comparatively short [22]. Downregulation of mRNA-binding showed that gene transcript process of the plant was inhibited under consecutive monoculture condition, which led to further influence on plant's physiology metabolism. Some proteins related to Calvin cycle, such as ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) kinase, Rubisco and Sedoheptulose-1,7-bisphosphatase (SBPase), etc. were down-regulated with the extended monoculture. Down-regulation of these proteins in the consecutively monocultured *R. glutinosa* affected its Calvin cycle, leading to lack of enough substances and energy necessary for various life activities during plant normal development, further causing the consecutive monoculture problems. Previous researches reported that SBPase gene could promote crop resistance by intensifying the capacity of starch biosynthesis and sugar production after transferred into plants for overexpression [23, 24]. So a new idea has been proposed that the method can be used for overcoming the *R. glutinosa* consecutive monoculture problems. Moreover, two kinds of fructose 1, 6-bisphosphate aldolases (spots 25 and 32), both play a key role similar to SBPase in sugar synthesis, catalyze the reversible conversion of triosephosphate into fructose 1, 6-diphosphate in leaf chloroplast (starch synthesis) and cytoplasm sucrose biosynthesis [25, 26]. Therefore, inactivation of these enzymes would greatly affect the smooth process of Calvin cycle, starch synthesis in chloroplast and sucrose biosynthesis in cytoplasm, leading to retarded plant growth.

By Infrared Spectroscopy and HPLC analysis, it showed that consecutive monoculture had no significant effect on the medicinal ingredients of *R. glutinosa*, but mainly caused the quantity of main medicinal ingredient (catalpol) to decline. The increment of peaks 2, 4 and decrement of catalpol peak area (peak 3) in SM compared to the NP indicated that

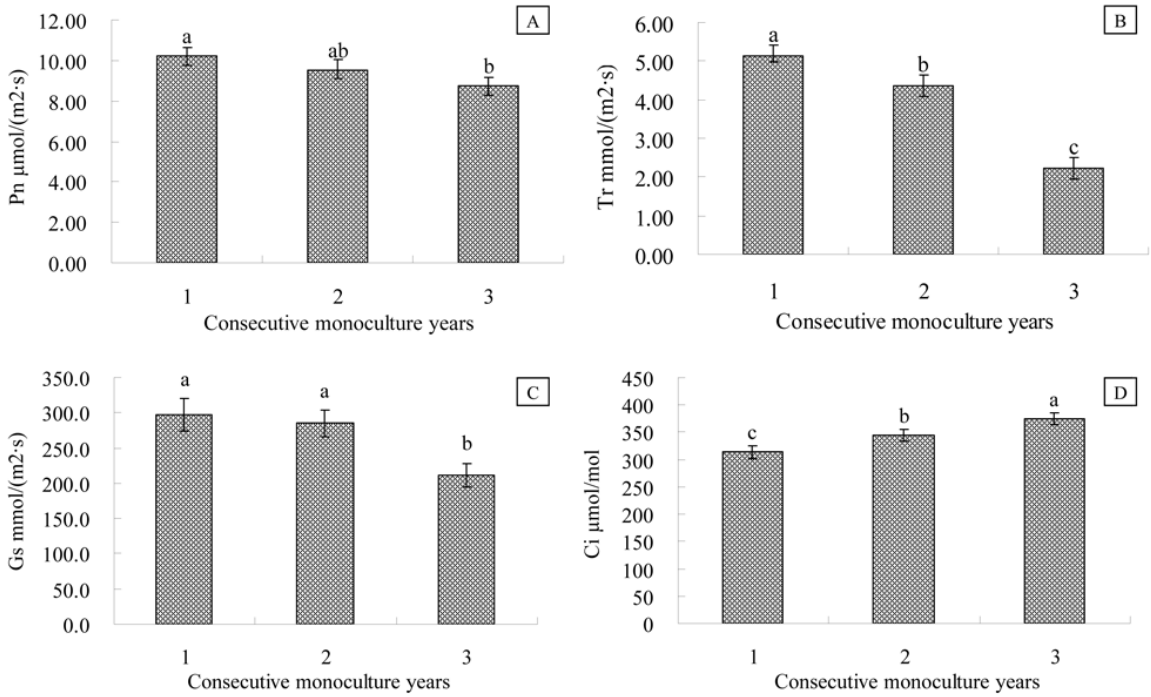

Figure 7. The effect of consecutive monoculture on net photosynthetic rate (A), transpiration rate (B), stomata conductance (C), and intercellular CO_2 concentration (D) of *R. glutinosa.* The numbers 1, 2 and 3 on the abscissa represent the plants from the newly planted (NP), the two-year (SM) and three-year consecutively monocultured (TM), respectively. Different letters show significant differences at the 5% level according to its p-value by Tucky's test (P≤0.05, n = 3).

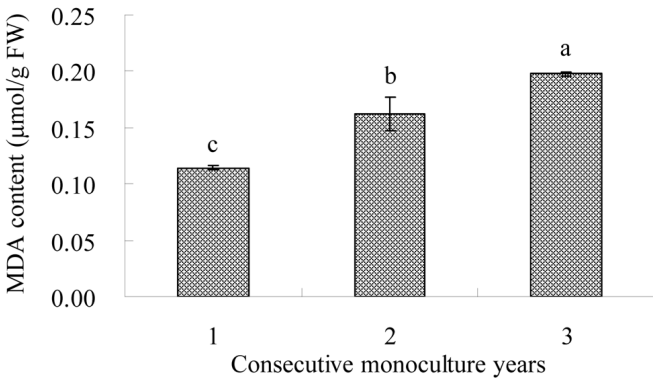

Figure 8. The effect of consecutive monoculture on MDA content in *R. glutinosa* leaves. The numbers 1, 2 and 3 on the abscissa represent the plants from the newly planted (NP), the two-year (SM) and three-year (TM) consecutively monocultured, respectively. Different letters show significant differences at the 5% level according to its p-value by Tucky's test (P≤0.05, n = 3).

catalpol, a kind of terpenes produced by acetate-malonate pathway (AA-MA pathway) in the plant body, was changed into peak 2-related and 4-related substances in the consecutively monocultured *R. glutinosa*. In this study, malonyl CoA -ACP transferase, one key enzyme in AA-MA pathway, was also down-regulated in the consecutively monocultured *R. glutinosa*, leading the decrease in catalpol biosynthesis. The above results showed that main medicinal ingredient- catalpol in *R. glutinosa* was greatly affected by the consecutive monoculture. Catalpol is one of the main active components of *R. glutinosa* and is found in the roots, stems and leaves [27,28,29].

One down-regulated protein (spot 27), related to pathogenesis, was identified in this study. Previous researches found that a few proteins were produced when plants were infected by virus, like-virus, fungus or bacterium, which were not pathogen-specific but were dependent on the type of host reaction [30]. These proteins united together in the infected site to form a protective barrier against pathogens invasion, reduce plant susceptibility to disease. It was suggested that these proteins might have the anti-fungus or anti -bacterium activity. Thus the pathogenesis-associated protein with down-expression found in the consecutively monocultured *R. glutinosa* in this study indicated that plant defense mechanisms might be damaged because of consecutive monoculture.

Protein degradation is important for normal development of plants, and proteasome plays an important role in the degradation of unwanted proteins when damaged [31]. This kind of degradation mechanism is necessary for many kinds of cell processes, including cell cycle, regulation of gene expression, oxidative stress reaction and so on. The main role of subunit α is to recognize a poly-ubiquitin chain which is connected to the target protein, and then initiates the corresponding degradation process. However, the protein (Proteasome subunit alpha type 5, spot 13) was down-regulated in consecutively monocultured *R. glutinosa*, which resulted in weak ability of adaptation. Besides, we also found that pyridoxine biosynthesis protein isoform A (spot 22) and Malonyl CoA-ACP transacylase (spot 10) were down-regulated in consecutively monocultured *R. glutinosa*. They were linked to the biosynthesis of pyridoxine, a coenzyme of many enzymes, and fatty acid [32].

From above differentially expressed proteins, it showed that consecutive monoculture had a negative effect on *R. glutinosa* basic biosynthesis (such as the synthesis of sugar, pyridoxine, fatty acid and proteins), the degradation of proteins and so on, then affected the secondary metabolism and disease defence. Thus the growth of plant was trapped into a vicious cycle, resulting in the decline of plant defense ability against outside adverse factors such as drought, high temperature, diseases, and insect pests and so on.

However, three proteins (spots 4, 21 and 33) related to the energy metabolism, such as ATP-binding protein, ATPase and ATP synthase subunit β, were all up-regulated in the consecutively monocultured *R. glutinosa*. It indicated that consecutively monocultured *R. glutinosa* was indeed threatened by adversity, which needed large amounts of energy to survive. Besides, two proteins (spots 14 and 35, i.e. heat shock proteins, HSPs,) linked to stress/defense response were up-regulated in the SM and TM compared with those in the NP. They play a crucial role in protecting plant body and normal development of cell, even in protein folding, subunit assembling, intracellular transportation , protein degradation and so on [33, 34, 35]. Two kinds of HSPs were up-regulated in the consecutively monocultured *R. glutinosa*, suggesting that the plant initiated many kinds of HSPs -related physiology and energy pathways to pull through the hard times, such as ATP-synthesis system, selective degradation of ATP-dependent proteins, when it was in unfavorable conditions.

In summary, the self-poisoning substances excreted by *R. glutinosa* were accumulated in the soil through consecutive monoculture, directly or indirectly affected the substance and energy metabolism, the stress/defense response, and some other regulations at molecular level in the plant body, which are necessary or important for the normal development of next cropping, such as microbe interaction, leaf photosynthesis, root activity, adversity resistance, production of main medicinal ingredients, crop yields and so on. So investigation of consecutive monoculture problems at the molecular level will deeply uncover the relevant mechanism, which contributes to find a molecular genetic way to improve the crop yield and the production of main medicinal ingredients of *R. glutinosa*, further promotes the development of Chinese medicinal industry.

5. Concluding Remarks

In this study, the physiological reactions and changes in leaf protein expression of *R. glutinosa* in response to monoculture years, as well as the main medicinal ingredients (catalpol) were determined. The results showed that consec-

utive monoculture remarkably inhibited the photosynthesis rate, root activity and the contents of chlorophyll and catalpol. Further research on the comparative proteomics displayed that proteins involved in Calvin cycle (i.e. Rubisco), AA-MA pathway (i.e. malonyl CoA-ACP transferase), pathogenesis and mRNA binding (i.e. chloroplast stem-loop binding protein-41), etc. were down-regulated with the increasing years of monoculture. However, proteins related to energy metabolism (i.e. ATPase and ATP synthase) and stress/defense response (i.e. heat shock protein) were highly up-regulated with the extended monoculture, which might be a response to environmental inhibitory factors (such as toxic microbial metabolites, low pH of soil and nutrient deficiency, etc.) under consecutive monoculture. In further study, a genetic engineering method may be used to regulate the expression of above-mentioned key enzymes in plant metabolism, offering an efficient way to alleviate the consecutive monoculture problems existing in *R. glutinosa* monoculture system.

Acknowledgements

This work was supported by grants from the National Science Foundation of China (Grant no. 30871494, 30772729) and the Natural Science Foundation of Fujian province (Grant no. 2008J0051).

References

1. Z.Y. Zhang, W.X. Lin, Chinese Journal of Eco-Agriculture. 17 (2009) 189-196. [in Chinese, English abstract.] DOI: 10.3724/SP.J.1011.2009.00189
2. A. Freddy, Agron. J. 93 (2001) 556-563.
3. C.H. Yang, D.E. Crowley, J.A. Menge, FEMS Microbiol. Ecol. 35 (2000) 129-136. DOI: 10.1111/j.1574-6941.2001.tb00796.x
4. A. Mithufer, Trends Plant Sci. 7 (2002) 440-444. DOI:10.1016/S1360-1385(02)02336-1
5. E.L. Rice, in: D.C. Washington (Ed.), Biochemical interactions among plants, National Academy of Sciences, USA, 1971, pp. 128-132.
6. J.F. Du, W.J. Yin, Z.Y. Zhang, J. Hou, J. Huang, J. Li, Chinese Journal of Ecology. 28 (2009) 445-450. [in Chinese, English abstract.]
7. Z.W. Wu, M.D. Wang, X.Y. Liu, H.G. Chen, X.C. Jia, Chinese Journal of Ecology. 28 (2009) 660-664. [in Chinese, English abstract.]
8. W.J. Yin, J.F. Du, J. Li, Z.Y. Zhang, China Journal of Chinese Materia Medica. 34 (2009) 18-21. [in Chinese, English abstract.]
9. X.S. Wen, S.L. Yang, J.H. Wei, J.H. Zheng, Chin Tradit Herbal Drugs. 33 (2002) 946-949. [in Chinese, English abstract.]
10. X.E. Li, S.L. Chen, S.Q. Wei, J.H. Wei, J. Lan, China Journal of Chinese Materia Medica. 31 (2006) 344-346. [in Chinese, English abstract.]
11. Z.F. Li, X.H. Qi, Q.S. Li, L.Q. Dai, M.Z. Lin, Z.Y. Zhang, W.X. Lin, Acta Ecologica Sinica. 30 (2010) 2576-2584. [in Chinese, English abstract.]
12. G. Jilani, S, Mehmood, A,N, Chaudhry, I. Hassan, M. Akram, Ann. Microbiol. 58 (2008) 351-357. DOI: 10.1007/BF03175528
13. L.K. Wu, H.B. Wang, Z.X. Zhang, R. Lin, Z.Y. Zhang, W.X Lin, PLoS ONE. 6 (2011) e20611. DOI: 10.1371/journal.pone.0020611
14. Y.H. Yang, X.J. Chen, J.Y. Chen, H.X. Xu, J. Li, Z.Y. Zhang, BMC Plant Biol. 11 (2011) 53. DOI: 10.1186/1471-2229-11-53
15. H.S. Li, Principles and techniques of plant physiological experiment, Higher Education Press, Beijing, 2000.
16. Q. Ling, W. Huang, P. Jarvis, Photosynth Res. 107 (2011) 209-214. DOI: 10.1007/s11120-010-9606-0
17. H. Esterbauer, K.H. Cheeseman, Methods in Enzymology B. 186 (1990) 407-421. DOI: 10.1016/0076-6879(90)86134-H
18. P.H. O'Farrell, J. Bio. Chem. 250 (1975) 4007-4021. DOI: 10.1007/978-1-60761-157-8_2
19. X.C. Wu, C.X. Fang, J.Y. Chen, Q.S. Wang, T. Chen, W.X. Lin, Z.L. Huang, J. Plant Biol. 54 (2011) 251-261. DOI: 10.1007/s12374-011-9162-y
20. H. Vorum, H. Hager, B.M. Christensen, S. Nielsen, B. Honoré, Exp. Cell Res. 248 (1999) 473-481. DOI: 10.1006/excr.1999.4431
21. A.C. Bergman, T. Benjamin, A. Alaiya, M. Waltham, K. Sakaguchi, B. Franzen, S. Linder, Electrophoresis. 21 (2000) 679-686. DOI: 10.1002/(SICI)1522-2683(20000201)21:3<679::AID-ELPS679>3.0.CO;2-A
22. J. Nielsen, J. Christiansen, J. Lykke-Andersen, A.H. Johnsen, U.M. Wewer, F.C. Nielsen, Mol. Cell. Biol. 19 (1999) 1262-1270. PMCID: PMC116055
23. L. Feng, Y. Han, G. Liu, B. An, J. Yang, G. Yang, Y. Li, Y. Zhu, Funct. Plant Biol. 34 (2007) 822-834. DOI: 10.1071/FP07074
24. L. Feng, K. Wang, Y. Li, Y. Tan, J. Kong, H. Li, Y. Li, Y. Zhu, Plant Cell Rep. 26 (2007) 1635-1646. DOI: 10.1007/s00299-006-0299-y
25. M. Tamoi, Y. Hiramatsu, S. Nedachi, K. Otori, N. Tanabe, T. Maruta, S. Shigeoka, Photosynth. Res. 108 (2011) 15-23. DOI: 10.1007/s11120-011-9645-1
26. J. Kossmann, U. Sonnewald, L. Willmitzer, Plant J. 6 (1994) 637-650. DOI: 10.1046/j.1365-313X.1994.6050637.x
27. G.C. Liu, H.Q. Du, L. Liang, Chinese Traditional and Herbal Drugs. 23 (1992) 71-73. [in Chinese, English abstract.]
28. C.H. Liu, G.S. Li, Y.X. Huang, Journal of Chinese Medicine Research. 14 (2001) 10-12. [in Chinese, English abstract.]
29. T.X. Wang, J.Y. Li, Z.H. Hu, Chinese Traditional and Herbal Drugs. 35 (2004) 208-209. [in Chinese, English abstract.]
30. F. Zhang, A. Simon, Virology. 312 (2003) 8-13. DOI: 10.1016/S0042-6822(03)00345-3
31. H. Lodish, A. Berk, P. Matsudaira, C.A. Kaiser, M. Krieger, M.P. Scott, S.L. Zipursky, J. Darnell, Molecular Cell Biology, fifth ed., WH Freeman, New York, 2004.
32. C. Oefner, H. Schulz, A. D'Arcy, G.E. Dale, Acta Crystallogr. D. Biol. Crystallogr. 62 (2006) 613-618. DOI: 10.1107/S0907444906009474
33. S. Lindquist, Annu. Rev. Biochem. 55 (1986) 1151-1191. DOI: 10.1146/annurev.bi.55.070186.005443
34. J.L. Key, C.Y. Lin, Y.M. Chen, Proc. Natl. Acad. Sci. USA. 78 (1981) 3526-3530. PMCID: PMC319602
35. W. Wang, B. Vinocur, O. Shoseyov, A. Altman, Trends Plant Sci. 9 (2004) 244-252. DOI: 10.1016/j.tplants.2004.03.006