INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
19–1804/202x/00–0–000–000
DOI: 10.17957/IJAB/15.1575
http://www.fspublishers.org

*Full Length Article*

# The Severe Infection of *Poacynum pictum* by Rust (*Melampsora apocyni*) Decreases the Flavonoids and Amino Acid Content and Affects Metal Concentrations in Plant Leaves

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*Received 25 November 2019; Accepted 07 January 2020; Published* _____

## Abstract

*Poacynum pictum* (Schrenk) Baill. is a perennial herbaceous plant, which is used as medicine and tea production. *Melampsora apocyni* Tranzschel. caused the rust disease which is mainly limiting factor for the plant growth. It is not clear whether the degree of infection caused by this disease will have effect on nutrient composition of leaves. The purpose of present work is to evaluate the effects of severity of the rust on biomass, total flavonoids, amino acid and metal content of two cultivated ecotypes of *P. pictum*, ecotype RSM with red stems of medium leaves (Eco–RSM) and ecotype GSF with green stems of fine leaves (Eco–GSF). When compared with healthy leaves, the biomass and total flavonoids of the GSF ecotype decreased due to the rust infection, but not statistically significant while severe rust significantly ($P < 0.05$) decrease the leaf biomass and flavonoids content of the RSM ecotype. The rust significantly ($P<0.05$) decreased the amino acid content in *P. pictum* leaves by severity 4 except for Methionine and Cystine in RSM ecotype plants, and Tryptophan for plants of GSF ecotype that remained at the same level as for rust-free leaves. The Ca and Cu amount in the plants of the RSM ecotype were decreased by the rust infection at severity 4 and 5; while on the other hand, the contents of these two metals in GSF ecotype plants were increased by severe rust. The extreme occurrence of rust led to decrease in leaf biomass and quality for tea and medicinal production, and is a limiting factor for the cultivation of *P. pictum*. 

**Keywords:** *Poacynum pictum*; Rust disease; Biomass; Flavonoids; Amino acid

## Introduction

*Poacynum pictum* from Apocynaceae family is widely distributed in Xinjiang China. There are more than 6 ecotypes of the species that can be distinguished from morphological and biological characteristics (Gao *et al.* 2015). *P. pictum* are perennial herbaceous plants and their heights can be more than 4 m. Plants leaves are used as a traditional Chinese medicine for the treatment of hypertension, hepatitis, and depression (Zhang 2004; Xie *et al*. 2012) and teas from the leaves of *P. pictum* have been used as a nutritional supplement in North American and East Asian health food markets (Shi *et al*. 2011; Song and Zhou 2015). The fiber obtained from the stem of the two species has become the interest in the textile industry (Gong *et al*. 2017).

The leaves of Apocynaceae plants contains high flavonoids content (Zhou *et al*. 2015) which has been considered to play a major role in Chinese traditional medicines and tea (Nishibe *et al.* 2001; Ma *et al.* 2003), *e.g*., flavonoids isolated from *Apocynum venetum* have significant anti-depressant activities for mice (Yan *et al.* 2015) and inhibit the progress of atherosclerosis in rats via the AMPK/mTOR pathway (Lü *et al*. 2017). Flavonoids have also been noted to play a role in suppressing the growth of bacteria (Kang *et al.* 2014), such as *Escherichia coli*, and highly decrease the ability of the bacteria to initiate an infection (Nguyen *et al.* 2016; Shang *et al.* 2017). In tissues, the substances may act as antioxidants and significantly contribute to scavenging of free radicals produced via metabolic processes in plants. The leaves of Apocynaceae plants contain metals such as Ca, Fe, Zn and Na which play important role in both plant and animal metabolism (Yokozawa *et al*. 2002). Rust caused by *Melampsora apocyni* is the major disease affecting *A. venetum* and *P. pictum*, and all ecotypes have been found to

be affected by this species causing rust (Gao *et al.* 2017a). The disease has been reported in Russia (Tranzschel 1891), Japan (Hiratsuka 1939), Kazakhstan (Nevodovskii 1956), China (Tai 1979), Bulgaria (Denchev 1995) and Turkey (Kirbağ 2004). In Xinjiang China, we studied the rust on leaves of wild and cultivated *A. venetum* plants from 2009 until now and this study is ongoing. The rust occurrence could reach up to 100% in field conditions cause the loss of leaves and also the death of plants in severe situations, ultimately causing large economic losses (Gao *et al.* 2017a).

Rust diseases have been reported to reduce the crop yield include *A. venetum* (Gao *et al.* 2017a), which decrease the crop protein and amino acid content in alfalfa and *Vicia sativa* (Nan 1986, 1990) and decrease the feed value of forage plants. Reports have revealed that rust diseases could decrease plant amino acid content by changing the metabolism of nitrogen in host plants (Nan 1986, 1990). Other reports found that rust pathogens such as species of *Melampsora*, *Phakopsora*, *Puccinia* and *Uromyces* can uptake amino acids in plants through intracellular haustoria, thus decrease amino acid content (Struck 2015). Rust disease has been reported to decrease the Ca and P concentration in alfalfa plants and these metals also play role in the plant disease defense system (Fones *et al*. 2010; Pinheiro *et al.* 2011). However, rust infection has been reported to enhanced the concentration of plant flavonoids (Miranda *et al.* 2007; Lu *et al.* 2017).

Two ecotypes of *P. pictum*, one with red stems with medium leaves referred to as ecotype RSM and another with green stems with fine leaves that is referred to as ecotype GSF, are essential cultivated plants in Altay for tea and fiber production (Gao *et al.* 2015). Rust has been found to be easily infect these two ecotypes, and the average disease incidence could reach up to 70% in cultivated fields. In this study, the effect of rust severity, based on a 6–step rating scheme and on series of parameters related to the quality of *P. pictum* for tea production was analyzed. We hypothesized that the infection of rust (*M. apocyni*) will decrease the biomass, amino acid content and metal concentration and increase the total flavonoids of these two ecotypes of *P. pictum*.

## Materials and Methods

### Experimental details and methods

**Samples cultivation:** The two ecotypes of *P. pictum*, Eco–RSM and Eco–GSF (Fig. 1), used in this study were basically obtained as selections from local naturally-growing *P. pictum* plants. They were cultivated in the Alakak Township in Altay Prefecture of the Xinjiang Uyghur Autonomous Region, China (Alakak field, 47°42′N, 87°33′E, at an altitude of 492 to 547 m, area 5.23 ha), using plants grown from seeds which were collected from locally growing plants. The soil was a sandy loam having pH of 7.2 to 7.5, and plant rows were spaced 3 meters apart while within the rows space was 1 m apart. Emergence of *P. pictum* stems began in the middle of April. *P. pictum* plants were irrigated by drip irrigation system after every 6 days and no disease control measures were applied.

### Rust disease survey

For each ecotype of *P. pictum*, four 20 × 20 m plots were used to assess the disease incidence of the rust species. From each plot 200 leaves were sampled non–destructively for each evaluation, and the disease incidence was recorded on leaves based on the presence of uredinia. The rust severity was recorded as a 6–step rating scheme by visually calculation of the percentage of observed leaves that were covered by uredinia: 0 for no signs of infection; 1 for 0.1 to 5% of leaf area covered with uredinia; 2 for 5.1 to 20%; 3 for 20.1 to 50%; 4 for 50.1 to 75%; and 5 for >75.1%. For each rust severity of the two *P. pictum* ecotypes (Fig. 2), 100 leaves of similar size from similar branches of the plants were sampled for dry weight, and total flavonoids, amino acid and metal content were measured.

### Total flavonoids extraction

According to this method, total flavonoids content (TFC) was calculated by acid hydrolysis method (Zhao *et al.* 2016) with little changes. For each rust severity, 100 leaves were dried in an oven at 65°C for 24 h, the dry leaves were ground into fine powders and mixed thoroughly. One-gram sample was precisely weighed and added to conical flask containing 25 mL of 70% ethanol in a water bath with 65°C constant temperature for 1h for reflux extraction. After cooling, 70% ethanol was added to the 25 mL and was thoroughly mixed and filtered through quantitative filter paper. One mL of the filtered solution was transferred to an evaporating dish, 1 g polyamide powder was added and the dish was kept in the water bath to remove the ethanol. The sample was then transferred into a chromatographic column, 20 mL of petroleum ether was added to remove impurities, the column was then washed with 20 mL of methyl alcohol and the eluent was collected. This eluent was allowed to dry. The residue was dissolved in methanol and transferred into a 10 mL volumetric flask. Two mL of the solution was added to 10 mL volumetric flask, and then added 5 mL of 30% ethanol and 0.3 mL of 5% sodium nitrite solution, and the solution was allowed to stand for 5 min. Then 0.3 mL of 10% nitric acid aluminum solution was added and the sample kept for another 6 min. Finally, 2 mL of 1.0 mol $L^{-1}$ sodium hydroxide solution was added. The absorbance was measured at 510 nm by ultraviolet visible absorption spectrophotometer (UV-7502c, China).

### Amino acid extraction

According to this method, amino acid extraction was measured by the Aluminium nitrate colorimetric method


**Fig. 1:** Eco RSM-red stem with medium leaves (left) and Eco GSF-green stem with fine leaves (right) of *P. pictum*

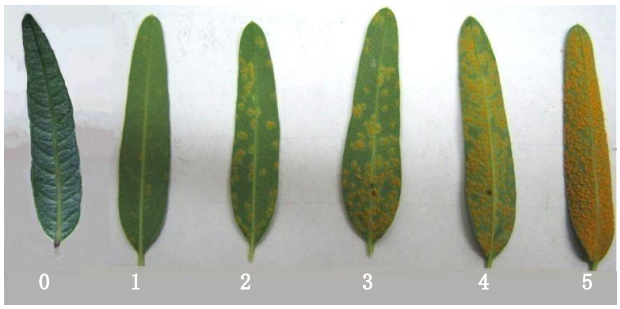


**Fig. 2:** The rust severity visually estimating the percentage of observed leaves that were covered by uredinia: 0 for no signs of infection; 1 for 0.1% to 5% of leaf area covered with uredinia; 2 for 5.1% to 20%; 3 for 20.1% to 50%; 4 for 50.1% to 75%; and 5 for >75.1%. For each rust severity of the two *P. pictum* ecotypes

with minor modification (Ksenofontov *et al*. 2017). The fine powder of *P. pictum* leaves as described in above method was used for amino acid extraction. For each rust disease severity, 30 mg powdered samples were weighed and added accurately to a hydrolysate tube in which 10 mL of 6 mol $L^{-1}$ hydrochloric acid was added. The hydrolysate tube was evacuated by a vacuum pump and then filled with nitrogen; this procedure was repeated 3 times and then the tube was sealed with an alcohol blast burner. The tubes with samples were kept for 22 h in a constant temperature oven at 110ºC to hydrolyze. The hydrolysate solutions were shifted to volumetric flasks and made up to constant volume of 50 mL with deionized water. One mL of the solution was added into a 5 mL of beaker and left to dry in glass desiccator. The dried sample was dissolved with 1 mL of acetic acid buffer having 5.5 pH, and used to detect the amino acids presence by use of an automatic amino acid analyzer (Hitachi 835, Japan).

Tryptophan was determined by alkaline hydrolysis method. Powdered samples (30 mg) were added accurately to a poly tetra fluoroethylene tube in which 3 mL of 5 mol $L^{-1}$ sodium hydroxide was added. The tubes with samples were kept for 22 h in a constant temperature oven at 110°C to hydrolyze. The hydrolysate solutions were shifted to 25 mL volumetric flasks and adjust pH to 7.6 after cooling, made up to constant volume of 25 ml with deionized water. Finally, 1 ml of the solution was shifted to 10 mL tube, made up to constant volume of 10 mL with 4 mol $L^{-1}$ (pH 11) urea solution, and used to detect the Tryptophan presence by use of a spectrofluorophotometer (Shimadzu RF-5000, Japan).

### Metal evaluation

For mineral content, 0.2 g of the dried samples were precisely weighed and dissolved in $HNO_3$:$HClO_4$:$H_2SO_4$ (8:1:1) mixture, and heated on a digestion furnace at 420°C in a fume hood. After the digestion and subsequent cooling, the digested samples were added to volumetric flasks and were diluted by the addition of ultrapure water to 100 mL. A flame atomic absorption spectrophotometer (Thermo ICE 3300, Germany) was used to measure metal elements (Zhang 2004).

### Statistical analysis

All data are presented as means and standard errors of means for four replicates. The significance of differences at a 5% level between averages was determined by one-way ANOVA using Tukey's HSD test.

## Results

### Rust disease occurrence, biomass production

In the research sites, the rust was firstly found in late July, and the two ecotypes of *P. pictum* had similar rust disease incidence during the growth period. The disease incidence was kept under 20% until the middle of August, and then there was a sharp increase from 20–60% in a week. Subsequently the development of rust disease slowed down but reached up to 70% in two weeks, and this persisted until leaves were lost and the growth period of the plants ceased (Fig. 3). The infection of rust had no effect on leaf dry weight of *P. pictum* plants of the GSF ecotype, however infection significantly decreased the dry weight of leaves of the RSM ecotype at rust severity 4 ($P < 0.05$). There was no significantly difference between rust-infected leaves with different rust severity (Fig. 4). Our hypothesis that rust will decrease the biomass of the two ecotypes of *P. pictum* was partly supported (Table 1).

### Total flavonoids concentration

The concentration of flavonoids in healthy leaves of the two ecotypes is 2.4–2.8 g 100 $g^{-1}$, which is 5.2–9.4% higher than rust–infected leaves. Compared with healthy leaves, the infection of the rust pathogen *M. apocyni* only significantly decreased flavonoids when the rust severity were 4 and 5 and the flavonoids concentrations were 36.4 and 25.5% lower than healthy leaves, respectively, for the RSM

**Table 1:** ANOVA result of the effects of rust disease caused by *Melampsora apocyni* on the listed variables to two ecotypes of *Poacynum pictum*

| Variables | Eco RSM -red stem with medium leaves | | | Eco GSF-green stem with fine leaves | | |
|---|---|---|---|---|---|---|
| | *F* values | *DF* | *P* values | *F* values | *DF* | *P* values |
| Leaf dry weight | 6.3478 | 5 | 0.0015 | 1.5728 | 4 | 0.2325 |
| Disease incidence | 18.6747 | 3 | 0.0001 | 22.0470 | 3 | 0.0001 |
| Flavonoids concentration | 3.9519 | 5 | 0.0136 | 0.4540 | 4 | 0.7681 |
| Calcium concentration | 4.4805 | 5 | 0.0079 | 5.2512 | 4 | 0.0076 |
| Copper concentration | 13.3846 | 5 | 0.0001 | 21.8334 | 4 | 0.0001 |
| Zinc concentration | 7.4581 | 5 | 0.0006 | 0.8233 | 4 | 0.5304 |
| Iron concentration | 3.7604 | 5 | 0.0166 | 2.3700 | 4 | 0.0991 |
| Alanine | 24.1229 | 5 | 0.0001 | 11.3179 | 4 | 0.0002 |
| Argnine | 34.1459 | 5 | 0.0001 | 16.0278 | 4 | 0.0001 |
| Aspartic | 21.9949 | 5 | 0.0001 | 5.3065 | 4 | 0.0072 |
| Cystine | 3.1365 | 5 | 0.0329 | 11.3432 | 4 | 0.0002 |
| Glutamine | 22.0609 | 5 | 0.0001 | 11.9762 | 4 | 0.0001 |
| Glycine | 26.2400 | 5 | 0.0001 | 9.6809 | 4 | 0.0004 |
| Hlstidine | 26.3829 | 5 | 0.0001 | 11.2136 | 4 | 0.0002 |
| Isoleucine | 24.0377 | 5 | 0.0001 | 9.7925 | 4 | 0.0004 |
| Leucine | 28.0698 | 5 | 0.0001 | 13.4100 | 4 | 0.0001 |
| Lysine | 21.4042 | 5 | 0.0001 | 11.5913 | 4 | 0.0002 |
| Methionine | 2.6925 | 5 | 0.0550 | 6.1619 | 4 | 0.0039 |
| Phenylalanine | 25.9132 | 5 | 0.0001 | 11.2994 | 4 | 0.0002 |
| Proline | 24.5499 | 5 | 0.0001 | 11.1099 | 4 | 0.0002 |
| Serine | 15.9173 | 5 | 0.0001 | 4.7319 | 4 | 0.0114 |
| Threonine | 22.2670 | 5 | 0.0001 | 9.9547 | 4 | 0.0004 |
| Tryptophan | 6.0371 | 5 | 0.0019 | 2.5727 | 4 | 0.0806 |
| Tyrosine | 44.6389 | 5 | 0.0001 | 14.4998 | 4 | 0.0001 |
| Valine | 24.2808 | 5 | 0.0001 | 10.0280 | 4 | 0.0004 |

ecotype (Fig. 5). There was no significant difference in flavonoid concentrations among leaves of all severities of leaves of the GSF ecotype (Fig. 5 and Table 1). Rust disease had no significant effects on flavonoids concentration in leaves of the GSF ecotype.

**Amino acid concentration**

Compared with healthy leaves, amino acid concentration was significantly affected (mainly decreased) by the rust disease except for methionine and cystine in the RSM ecotype, and tryptophan in the GSF ecotype. The decreases in the concentration of these three amino acids happen under all rust disease severity (Table 1 and 2). *P. pictum* leaves with rust severity 4 and 5 had the lowest amino acid concentration for the RSM ecotype. For the GSF ecotype the content of 10 and 16 of 18 amino acids was reduced by rust disease at rust severity 3 and by the rust disease at severity 4, respectively, rust severity 4 had significantly higher amino acid content, 16 of 18 except Methionine and Trytophan, than that of rust severity 1. When rust severity were 4 and 5, rust disease significantly decreased the concentration of 15 amino acids in leaves of the RSM ecotype, Except for Cystine, Methionine and Trypothan, which given the same value under the different rust severity (Table 2).

The correlations of rust disease severity and the amino acids depicts that amino acid content is negatively correlated with rust disease severity for the two ecotypes, except for Cystine, Methionine and Trytophan (Table 3). The amino acid response to rust disease differed in the two ecotypes. With the GSF ecotype the content of Methionine and Trytophan had no correlation with rust disease severity. Glutamine is the most sensitive amino acid to rust disease, followed by Leucine, while Trytophan is the least sensitive amino acid to rust disease with both ecotypes of *P. pictum* (Table 2). Our hypothesis that rust will decrease the amino acid of the two ecotypes of *P. pictum* was upheld.

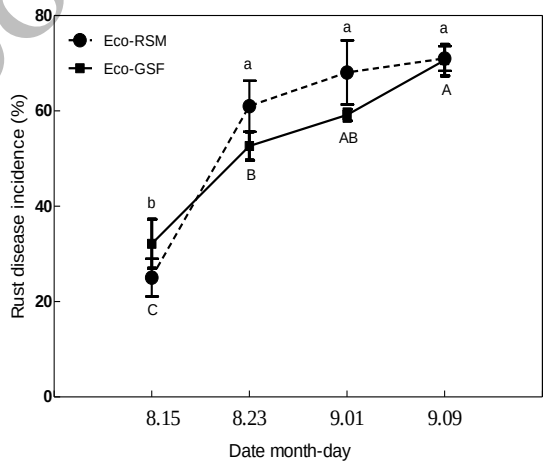


**Fig. 3:** Rust disease incidence of two ecotypes Eco RSM-red stem with medium leaves and
Eco–GSF green stem with fine leaves of *P. pictum* in cultivated field. The same lowercase letters up the bars means there is no significantly different by Turkey's HSD at $P < 0.05$ for Eco RSM; The same uppercase letters up the bars means there is no significantly different by Turkey's HSD at $P < 0.05$ for Eco GSF

**Table 2:** Amino acid concentration of two ecotypes of *Poacynum picttum* under different rust disease severity caused by *Melampsora apocyni* %

| Amino acid | Rust disease severity of Ecotype red stem with medium leave (Eco-RSM) | | | | | | Rust disease severity of Ecotype green stem with fine leaves (Eco-GSF) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 0 | 1 | 2 | 3 | 4 |
| Alanine | 1.22±0.05 a* | 1.24±0.05 a | 1.28±0.01 a | 1.11±0.06 a | 0.82±0.05 b | 0.77±0.04 b | 1.31±0.07 a | 1.23±0.05 ab | 1.04±0.04 bc | 1.08±0.06 ab | 0.84±0.05 c |
| Argnine | 1.16±0.05 ab | 1.19±0.05 ab | 1.34±0.06 a | 1.01±0.05 b | 0.73±0.04 c | 0.68±0.03 c | 1.20±0.07 a | 1.10±0.05 ab | 0.93±0.01 bc | 0.92±0.03 bc | 0.73±0.05 c |
| Aspartic | 1.76±0.08 a | 1.86±0.08 a | 1.97±0.05 a | 1.68±0.08 a | 1.24±0.07 b | 1.18±0.05 b | 1.87±0.10 a | 1.73±0.07 a | 1.55±0.07 ab | 1.60±0.10 ab | 1.32±0.10 b |
| Cystine | 0.58±0.06 a | 0.55±0.02 a | 0.49±0.01a | 0.47±0.05 a | 0.39±0.03a | 0.46±0.03a | 0.62±0.02 a | 0.56±0.00ab | 0.51±0.01bc | 0.48±0.03bc | 0.46±0.02 c |
| Glutamine | 2.41±0.08 a | 2.44±0.13 a | 2.39±0.01 a | 2.21±0.13a | 1.59±0.07 b | 1.53±0.07 b | 2.46±0.12 a | 2.26±0.09 ab | 1.97±0.03 bc | 1.97±0.08 bc | 1.65±0.10 c |
| Glycine | 1.04±0.05 a | 1.08±0.05 ab | 1.15±0.02 ab | 0.95±0.04 b | 0.70±0.04 c | 0.66±0.03 c | 1.12±0.06 a | 1.04±0.05 ab | 0.90±0.02 bc | 0.93±0.05 abc | 0.74±0.04 c |
| Hlstidine | 0.45±0.02 ab | 0.46±0.02 ab | 0.51±0.02 a | 0.41±0.02 b | 0.30±0.02 c | 0.28±0.02 c | 0.48±0.02 a | 0.44±0.02 ab | 0.38±0.01 bc | 0.40±0.02 ab | 0.30±0.02 c |
| Isoleucine | 0.96±0.04 ab | 1.00±0.04 ab | 1.07±0.02 a | 0.89±0.04 b | 0.67±0.04 c | 0.64±0.03 c | 1.00±0.06 a | 0.96±0.04 ab | 0.82±0.02 bc | 0.83±0.03 abc | 0.69±0.04 c |
| Leucine | 1.73±0.08 ab | 1.81±0.08 ab | 1.96±0.04 a | 1.56±0.08 b | 1.14±0.07 c | 1.07±0.06 c | 1.88±0.11 a | 1.75±0.08 ab | 1.47±0.03 bc | 1.48±0.05 bc | 1.19±0.07 c |
| Lysine | 1.21±0.04 a | 1.23±0.05 a | 1.18±0.02 a | 1.09±0.06 a | 0.80±0.05 b | 0.76±0.04 b | 1.23±0.07 a | 1.15±0.05 ab | 1.01±0.01 bc | 1.02±0.03 bc | 0.83±0.04 c |
| Methionine | 0.21±0.06 a | 0.08±0.00 a | 0.09±0.00 a | 0.08±0.01 a | 0.12±0.04 a | 0.10±0.02 a | 0.13±0.00 ab | 0.16±0.02 ab | 0.18±0.02 a | 0.13±0.01 b | 0.11±0.00 b |
| Phenylalanine | 1.10±0.05 ab | 1.16±0.05 ab | 1.29±0.04 a | 1.03±0.05 b | 0.75±0.04 c | 0.70±0.04 c | 1.20±0.07 a | 1.13±0.05 ab | 0.97±0.02 bc | 0.98±0.04 bc | 0.80±0.04 c |
| Proline | 0.92±0.03 a | 0.95±0.04 a | 1.00±0.02 a | 0.86±0.04 a | 0.63±0.03 b | 0.60±0.03 b | 0.98±0.05 a | 0.92±0.04 ab | 0.82±0.03 bc | 0.80±0.03 bc | 0.68±0.03 c |
| Serine | 0.81±0.04 a | 0.83±0.03 a | 0.80±0.01 a | 0.74±0.04 a | 0.58±0.03 b | 0.55±0.03 b | 0.81±0.04 a | 0.83±0.03 a | 0.80±0.01 a | 0.74±0.04 a | 0.58±0.03 b |
| Threonine | 0.91±0.04 a | 0.96±0.04 a | 0.96±0.00 a | 1.68±0.04 a | 0.63±0.03 b | 0.59±0.03 b | 0.98±0.05 a | 0.90±0.04 ab | 0.79±0.02 bc | 0.81±0.04 bc | 0.66±0.04 c |
| Tryptophan | 0.13±0.01ab | 0.17±0.01 a | 0.14±0.01 a | 0.16±0.03 a | 0.07±0.01 b | 0.12±0.02 ab | 0.13±0.00 a | 0.12±0.00 a | 0.18±0.02 a | 0.16±0.02 a | 0.17±0.01a |
| Tyrosine | 0.68±0.01 a | 0.65±0.02 a | 0.68±0.00 a | 0.53±0.03 b | 0.39±0.03 c | 0.37±0.01 c | 0.70±0.04 a | 0.65±0.03 ab | 0.53±0.01 bc | 0.54±0.02 bc | 0.42±0.03 c |
| Valine | 1.21±0.04 a | 1.28±0.06 a | 1.35±0.03 a | 1.13±0.05 a | 0.84±0.05 b | 0.80±0.04 b | 1.28±0.07 a | 1.21±0.06 ab | 1.06±0.04 bc | 1.05±0.04 bc | 0.87±0.05 c |

Note:* Data marked by the same lowercase letter in the same row do not differ significantly between rust severity for the same ecotype of *Pocynum pictum.*

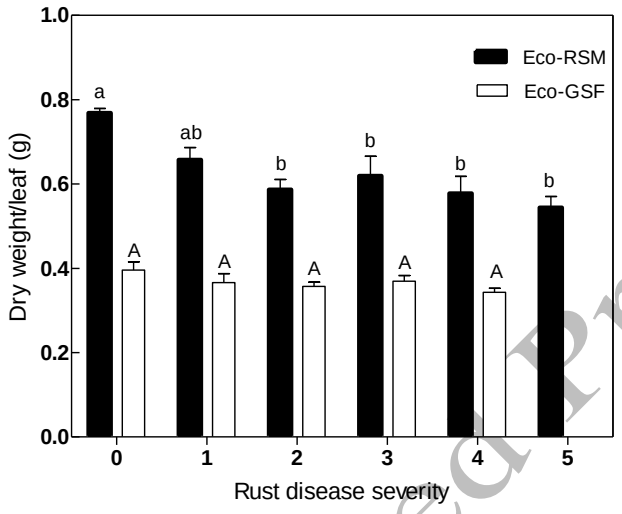


**Fig. 4:** Leave dry weight of two ecotypes Eco RSM-red stem with medium leaves and Eco
GSF-green stem with fine leaves of *P. pictum* in cultivated field. The same lowercase letters
up the bars means there is no significantly different by Turkey's HSD at $P<0.05$ for Eco RSM;
The same uppercase letters up the bars means there is no significantly different by Turkey's HSD at $P<0.05$ for Eco GSF

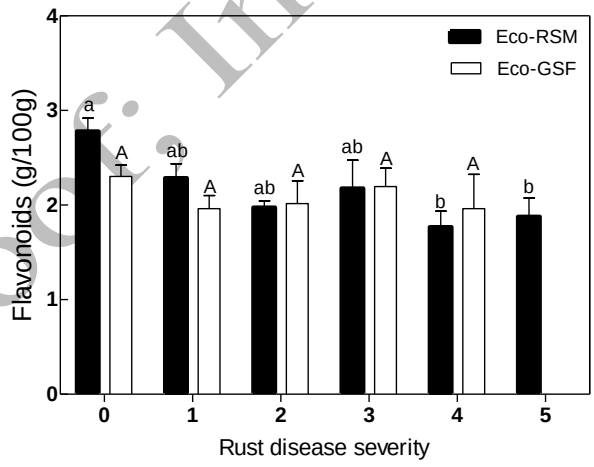


**Fig. 5:** Flavonoids of two ecotypes Eco RSM-red stem with medium leaves and Eco GSF-green stem with fine leaves of *P. pictum* in cultivated field. The same lowercase letters up the bars
means there is no significantly different by Turkey's HSD at $P < 0.05$ for Eco RSM; The same
uppercase letters up the bars means there is no significantly different by Turkey's HSD at $P < 0.05$ for Eco GSF

## Metal concentration

Rust show different effects on calcium (Ca), copper (Cu), iron (Fe) and zinc (Zn) concentrations of the two ecotypes of *P. pictum* (Table 1). For the GSF ecotype, rust disease had no effect on Fe and Zn concentration, while in case of severe rust infection (severity 4) significantly ($P < 0.05$) increased Ca and Cu concentrations. For the RSM ecotype, when compared with healthy leaves, the concentration of Ca was significantly ($P < 0.05$) decreased at rust severity 5, and with Cu at rust severity 4 and 5. Healthy leaves had the same concentration of Zn and Fe as with rust-infected leaves, but there was significant difference among rust-infected leaves with varying rust severity. Rust severity 2 and 3 had higher Zn concentrations than the other severities of rust infection. Rust severities of 5 and 3 had the highest and lowest Fe concentration among rust-infected leaves (Fig. 6). Our hypothesis that rust will decrease the metal of the two ecotypes of *P. pictum* was partly upheld.

## Discussion

Our previous research showed that infection by *M. apocyni* causes the leaves of *A. venetum* to turn yellow, wither and prematurely fall, and results in significant yield loss in the field (Gao *et al*. 2015). In a greenhouse experiment, infection by *M. apocyni* had a slight effect on photosynthesis of *A. venetum* during early disease

**Table 3:** Correlations of rust disease severity caused by *Melampsora apocyni* and the listed variables to two ecotypes of *Poacynum hendersonii*

| Variables | Eco RSM-red stem with medium leaves | | | Eco GSF-green stem with fine leaves | | |
|---|---|---|---|---|---|---|
| | Coefficient | Regression equation | SE | Coefficient | Regression equation | SE |
| Leaf dry weight | 0.710** | Y=0.721-0.038x | 0.067 | 0.453 | Y=0.387-0.010x | 0.030 |
| Flavonoids concentration | 0.621** | Y=2.563-0.168x | 0.378 | 0.150 | Y=2.177-0.044x | 0.435 |
| Alanine | 0.820** | Y=1.336-0.105x | 0.131 | 0.819** | Y=1.319-0.109x | 0.114 |
| Argnine | 0.790** | Y=1.314-0.118x | 0.164 | 0.879** | Y=1.202-0.113x | 0.092 |
| Aspartic | 0.757** | Y=1.974-0.144x | 0.222 | 0.722** | Y=1.895-0.122x | 0.174 |
| Cystine | 0.589 | Y=0.566-0.030x | 0.074 | 0.852** | Y=0.606-0.039x | 0.036 |
| Glutamine | 0.836** | Y=2.601-0.203x | 0.238 | 0.851** | Y=2.444-0.192x | 0.177 |
| Glycine | 0.796** | Y=1.163-0.093x | 0.126 | 0.804** | Y=1.118-0.086x | 0.095 |
| Hlstidine | 0.772** | Y=0.503-0.041x | 0.060 | 0.812** | Y=0.477-0.039x | 0.042 |
| Isoleucine | 0.775** | Y=1.066-0.079x | 0.115 | 0.817** | Y=1.010-0.076x | 0.080 |
| Leucine | 0.790** | Y=1.956-0.164x | 0.227 | 0.856** | Y=1.884-0.165x | 0.148 |
| Lysine | 0.850** | Y=1.304-0.103x | 0.114 | 0.837** | Y=1.234-0.094x | 0.092 |
| Methionine | 0.332 | Y=0.147-0.013x | 0.068 | 0.350 | Y=0.158-0.008x | 0.333 |
| Phenylalanine | 0.748** | Y=1.257-0.100x | 0.159 | 0.837** | Y=1.206-0.096x | 0.093 |
| Proline | 0.794** | Y=1.020-0.038x | 0.106 | 0.846** | Y=0.982-0.071x | 0.067 |
| Serine | 0.827** | Y=0.087-0.061x | 0.074 | 0.613** | Y=0.860-0.051x | 0.098 |
| Threonine | 0.810** | Y=1.006-0.076x | 0.098 | 0.814** | Y=0.977-0.074x | 0.079 |
| Tryptophan | 0.387 | Y=0.154-0.009x | 0.040 | 0.446 | Y=0.132+0.010x | 0.029 |
| Tyrosine | 0.895** | Y=0.726-0.071x | 0.063 | 0.861** | Y=0.704-0.067x | 0.059 |
| Valine | 0.776** | Y=1.353-0.101x | 0.146 | 0.829** | Y=1.289-0.098x | 0.098 |
| Caconcentration | 0.555* | Y=4.727-0.323x | 0.864 | 0.610* | Y=8.563-0.910x | 1.763 |
| Cu concentration | 0.816** | Y=0.186-0.025x | 0.032 | 0.639* | Y=0.132+0.070x | 0.126 |
| Zn concentration | 0.384 | Y=0.790-0.055x | 0.238 | 0.374 | Y=2.142-0.180x | 0.667 |
| Fe concentration | 0.235 | Y=0.051+0.007x | 0.049 | 0.594* | Y=0.166-0.022x | 0.045 |

Note:SE=standard error; **P* < 0.05, ***P* < 0.01; x=rust disease severity.

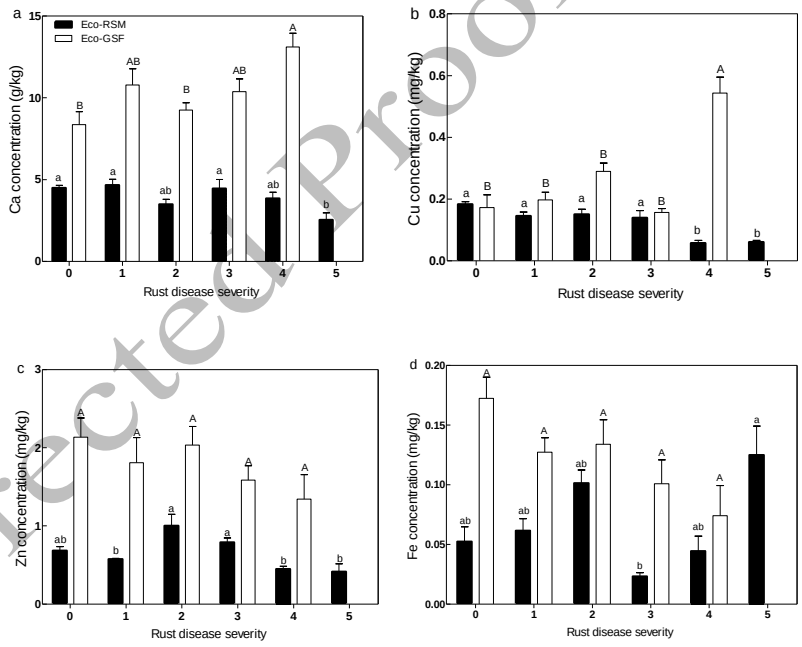


**Fig. 6:** Metal concentration of two ecotypes Eco RSM-red stem with medium leaves and Eco GSF-green stem with fine leaves of *P. pictum* in cultivated field. The same lowercase letters up the bars means there is no significantly different by Turkey's HSD at *P*<0.05 for Eco RSM; The same uppercase letters up the bars means there is no significantly different by Turkey's HSD at *P*<0.05 for Eco GSF

development, but drought stress was more damaging than for non-inoculated plants in later disease development, leading to a great decrease in the net photosynthetic rate This reduction, however, did not cause a significantly decrease in the aboveground biomass of *A. venetum* plants between the rust-infected and non-infected treatments (Gao *et al.* 2017b). The difference in leaf biomass of the two ecotypes of *P. pictum* following rust infection indicates the diversity of plant responses to this pathogen.

Several research studies have found that the infection by pathogens increased plant flavonoids concentration in plant tissues. An example of this was that Miranda *et al.*

(2007) using the Populus 15.5K cDNA microarray, found that genes encoding enzymes required for synthesis of the flavonoid proanthocyanidin were up regulated dramatically. Phytochemical analysis confirmed that in late infection, proanthocyanidin levels increased in infected leaves. Lu *et al*. (2017) also found that the amount of flavonoid compounds, especially anthocyanin and catechin, were significantly increased in rust-infected symptomatic tissue. The expression levels of structural genes and MYB transcription factors related to flavonoid biosynthesis were one to seven-fold higher in the tissue infected by rust.

The present study indicates that the two ecotypes of *P. pictum* had different responses to rust infection, as rust had no effects on flavonoids content of leaves of the GSF ecotype |but resulted in decreased flavonoids concentration in the leaves of the RSM ecotype. This decrease with the RSM ecotype is opposite to previous reports about the effects of rust disease on plant flavonoids concentration. The dry weight and flavonoids of the two ecotypes of *P. pictum* had the same response to rust. The accumulation of carbohydrates in rust-infected plants may be associated with the flavonoid biosynthesis pathway (Wan *et al.* 2015) and also, those carbohydrates which are a component of osmotic regulation during pathogen infection, may contribute to the accumulation of flavonoids (Lu *et al.* 2017). This new finding that rust decreases *P. pictum* flavonoids is important supplementary knowledge of the effects of rust disease on plant flavonoids as well as the evaluation of the resulting loss. It helps the understanding of physiological effects of this rust species as found in our previous study in a greenhouse that showed that the infection of rust changed activity of peroxidase, polyphenol oxidase and phenylalanine ammonialyase in *A. venetum* leaves (Gao *et al*. 2017b). Our hypothesis that rust will increase the total flavonoids of the two ecotypes of *P. pictum* was not upheld.

Many reports depicted that rust disease decreases the amino acid content of plants. For example, Nan found that the infection of rust (*Uromyces onobrychidis*) in *Onobrychis viciaefolia*, *U. orobi* in *Vicia sativa* and *U. striatus* in alfalfa (*Medicago sativa*) and *U. baeumlerianus* in *Melilotus albus*, decreased total crude protein and 16 amino acids by more than 30% (Nan 1986, 1990). Rust fungi only can complete their life cycle on living hosts where they grow through the leaf tissue by developing an extended network of intercellular hyphae from which intracellular haustoria are involved in suppressing host defense responses and acquiring nutrients. Three amino acid transporter genes of the rust fungi, Uf–*AAT1*, Uf–*AAT2* and Uf–*AAT3*, are closely related with intracellular haustoria of rust fungi. AAT1 and AAT3 are expressed very early during rust development and are strongly up-regulated in haustoria (Struck *et al*. 2002, 2004) while AAT2 was shown to be strictly haustorium specific (Hahn and Mendgen 1997). The decrease in amino acid content with severe infection of rust disease in the two ecotypes of *P. pictum* may partly be due to the regulation of the expression of these three transporter genes by the rust pathogen, as well as the consumption, metabolism or the storage of amino acid by the pathogens (Hahn and Mendgen 1997; Struck *et al*. 2002, 2004). There is also research found that pathogens such as *Pseudopeziza medicaginis* in alfalfa decrease the content of amino acids in host plants (Morgan and Parbery 1980). Even a low level of infection by *Drechslera siccans* or *Rhynchosporium* spp. significantly reduced *in vitro* dry matter digestibility, and water-soluble carbohydrate and the total amino acid content of Italian ryegrass (*Lolium multiflorum)* and tall fescue (*Festuca arundinacea*), and the decreases are correlated with the nitrogen metabolism and transportation in the plant–pathogen system.

Essential and non–essential heavy metals, such as Cu and Zn, are quantified in selected medicinal plants, including *A. venetum* and *P. pictum*, which are extensively used in the preparation of herbal medicines for heart disease and tonics for general human health. Rust disease has been shown decreased the content of Ca and P in alfalfa (Nan 1986), but the mechanism is not clear. The defensive properties of metals against diseases of plants has received much attention and support (Fones *et al.* 2010; Fones and Preston 2013), an example of which is the supply of Ca and K reduces soybean rust (*Phakopsora pachyrhizi*) area under the disease progress curve (PUDPCS) (Pinheiro *et al.* 2011). The accumulation of metals in plants is correlated with the activity of metal transporters, *e.g*., repeated duplication of the gene encoding the P–type ATPase, HMA4, which is responsible for xylem loading of Zn and cadmium (Cd) (Hanikenne *et al*. 2008).

## Conclusion

Rust disease was caused by *Melampsora apocyni* widely occur in field of cultivated *P. pictum*, and the disease incidence could reach up to 70% for the two ecotypes. The severe occurrence of rust disease led to the decrease of leaf biomass and flavonoids in the RSM ecotype, reduced the amino acid content of the two ecotypes, and increased or decreased Ca and Cu for the GSF and RSM ecotypes, respectively. These changes due to rust infection decreased the value of *P. pictum* for tea and Chinese traditional medicine production. Control methods for this rust disease are urgently required in this region.

## Acknowledgements

This research was financially supported by Key Project of Science and Technology Department of Xinjiang Autonomous Region, China (2016E02015, 2016A03006).

## Author Contributions

Tingyu Duan and Peng Gao designed the experiment and analyzed the data; Yanru Lan performed the experiments; Tingyu Duan wrote the manuscript.

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