

Inhibitory Activity of Oak Pyroligneous Liquor against *Coleosporium Plectranthi*, an Obligate Parasite Responsible for the Rust Disease on Perilla Leaf

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Abstract

BACKGROUND: *Coleosporium plectranthi*, an obligate parasite, which is responsible for the rust disease of *Perilla frutescens*, a plant in Korea, commonly known as Perilla. All rusts are obligate parasites, meaning that they require a living host to complete their life cycle. They generally do not kill the host plant but can severely reduce growth and yield. Food and feed spoilage fungi cause great economic losses worldwide. It is estimated that between 5 and 10% of the world food production is wasted due to fungal deterioration. Rust disease of Perilla is highly frequent and is widely spread in Korea. The present study was designed to investigate a novel media for the urediniospore germination *in vitro* and anti-rust activity as well as GC-MS analysis of oak pyroligneous liquor.

METHOD AND RESULTS: Urediniospores were collected from the infected leaf of Perilla. Spore suspension was made and the suspension was inoculated in the 2% water agar media with proper humidity, then they were incubated at 26°C for 56 hrs. The GC-MS analysis of the oak pyroligneous liquor was also done to check the chemical composition. GC-MS analysis of the wood vinegar was found 15 compounds, among them o-methoxyphenol (25.93%), 2,6-dimethoxyphenol (16.06%), 4-methylenecyclohexanone (10.69%), 2,3-dihydroxytoluene (7.84%), levoglucosane (6.14%) and

propanoic acid (5.32%) were the major components. Different concentration of the oak pyroligneous liquor was used, and spore inhibition was recorded on the basis of spore counting. The best results were noted at the concentration of 50% solution where 31.8% spores were inhibited.

CONCLUSION: On the basis of the chemical composition of the oak pyroligneous liquor and the activity recorded we can use it as an anti-rust agent.

Key Words: *Coleosporium plectranthi*, GC-MS, Oak pyroligneous liquor, Perilla

INTRODUCTION

The genus *Coleosporium* belongs to the family *Coleosporaceae* of the order *Uridinales*. This family has two other genera and nearly 80 cosmopolitans including the genus *Coleosporium*. The genus has numerous described species, many of which are doubtfully distinct morphologically (Cummins, 1997).

Historically, the classification of *Coleosporium* was decided by the morphology of teliospores, and species was decided by alternate hosts. Nevertheless recent found that the *Coleosporium species* are not strict in selecting for alternate hosts of some species often overlaps (Sato and Sato, 1982).

Perilla frutescens commonly known as Perilla is an annual herb of the genus *Perilla* of the mint family, *Lamiaceae*. Perilla is a summer annual plant and adapted to warm humid climates. The seed can be

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planted one centimeter deep as early as possible in the spring. The flowers self-pollinate without insect visits (Brenner, 1993). It is also known as beefsteak plant. Its essential oil has a strong taste. Its foliage is widely used as a side dish in Korean food and seed oil are also used in Korean cooking. The foliage is used as potherb and a garnish in Japan as well. The seed are eaten in Japan, Korea and India (Brenner, 1995). In Japan the foliage also provides a red (anthocyanin) food coloring and specialized red leaved perilla varieties are used in production of pickled plums (Suyama *et al.*, 1983). Perilla is also used in Oriental medicine, especially in China (Chen 1997). The entire plant is very nutritious, packed with vitamins, minerals, and a variety of chemical components (Kurita & Koike 1981; Fujita & Nakayama 1997; Ueda & Yamazaki 1997; Ragazinskiene *et al.* 2004). It has been found that seed oil is rich in omega-3 fatty acid (alpha linolenic acid) which has some benefit in the treatment of allergy (Choi *et al.*, 1980; Yu *et al.*, 1997; Baser *et al.*, 2003).

Rust disease of Perilla is highly frequent and is widely spread in Korea (Al-Reza *et al.*, 2010). End of June to mid August is the probable time when the Perilla rust occurs during the growing seasons. Initially, the symptoms appear as tiny yellowish projections on the lower surface of the leaves usually spreading inwardly from terminal leaf edge, accompanied by yellow to brown flecks formed on their opposite upper side and covering the whole leaves with spore masses within 2-3 weeks. Both aeciospores and urediniospores are reportedly pathogenic to Perilla, which are known to serve primary and secondary inocula, respectively, in a macrocyclic disease cycle of the rust fungus. However, at present the involvement of aeciospores in the development of Perilla rust may be relatively low because Perilla is widely grown in greenhouses in which the disease is prevalent during the wintertime and urediniospores readily over winter to serve as the continuous secondary inocula in the next year (as primary inoculum). Also the urediniospores must be the inoculum of the secondary infection responsible for rust epidemic in Perilla (Yun *et al.*, 2007).

An Oak is a tree or shrub in the genus *Quercus*, of which about 600 species exist on earth. "Oak" may also appear in the names of species in related genera, notably *Lithocarpus*. The genus is native to the northern hemisphere and includes deciduous and evergreen species extending from cold latitudes to

tropical Asia and the Americas.

Most parts of the Oak tree are used medicinally and their healing effects are varied. The distilled water of the Oak leaf bud can be taken internally or used externally to relieve minor inflammations. Bruised Oak leaves applied externally to wounds and hemorrhoids will also help to reduce and ease inflammation. The bark of the Oak tree is part most used in medicine, it being a tonic, astringent and antiseptic. As with other astringents it is also recommended for use in agues and hemorrhages. When bark is boiled with water, it can then be taken in a wineglass measure or dose, and used as a gargle mouthwash for chronic sore throats, or applied locally to bleeding gums and piles. It is also used in hot baths for chilblains and frostbite or as a hot compress for inflamed glands, hernias and hemorrhoids. A stronger decoction taken by the spoonful is useful in chronic diarrhea.

Oak bark as a powder makes a remedial snuff that can be inhaled to arrest nosebleeds. A pinch of powdered Oak bark mixed with honey and taken in the morning will help and aid ladies with menstrual problems. Ground and powdered acorns taken with water was considered as a useful tonic for diarrhea, and a decoction of acorns and Oak bark made with milk, was used as an antidote to poisonous herbs and medicines. In old times, the thin skin of acorn was used to cover open cuts or wounds, and ground and powdered acorns taken in wine was considered as a good diuretic (George Knowles, 2002).

Materials and Methods

Collection of the samples

The leaves of Perilla infected by *C. plectranthi* were collected freshly from the open field near Daegu University, Kyongsan, Republic of Korea in August 2011. The infected leaves with different size of pustules which are visibly free from other contaminants were selected and the urediniospores were collected in a gelatin capsule by scooping the spores from each pustules of the leaf formed the bulk sample.

Preparation of urediniospore suspension

Urediniospores were mixed in 25 ml of sterile distilled water, pre-added with one small drop of 0.01% Tween 20 to make the final spore concentration approximately 1×10^5 spores / ml.

Preparation of agar coated slide

Agar coated slides were prepared, under sterile conditions, by pipetting approximately 4 ml of 2% water agar on each slide. Triplicates slides for each treatment were prepared. To each prepared slide, 100 μ l of the urediniospore suspension was added and spread uniformly with help of sterile spreader on the agar medium.

In vitro culture of rust spore

For *in vitro* culture of rust spore saturated salts were prepared and poured into Petri dishes that had been lined with five layers of tissue paper (KimtechTM) to soak up the saturated salt solution and to prevent spillage. Potassium Chloride (KCl), Potassium nitrate (KNO₃) and water were used to achieve the proper humidity (Greenspan, 1977). Then the plates were incubated at 26°C for 56 hrs.

Observation and calculation of spores

After completing the desired incubation period the slides laden with urediniospores were observed under the compound microscope (Nikon Alphaphot 2, Japan) at 100X oil immersion magnification. Per cent urediniospore was calculated using the formula (Kamanna *et al.*, 1993)

$$PGU = \frac{TNGU}{TNU} \times 100$$

PGU = Percent germinated urediniospores,

TNGU = Total no. of germinated urediniospores in each microscopic field,

TNU = Total no. of urediniospores present in each microscopic field.

Scanning Electron Microscopic (SEM) Analysis

Scanning electron microscopy (SEM) of germinating urediniospores was done. For the preparation of SEM samples, the slides laden with urediniospores were cut in small sections and these were fixed overnight at 4°C in 4% glutaraldehyde in 0.05 M phosphate buffer (pH 7.3) and washed three times (10min. each) in phosphate buffer. After this 10 min. rinses in distilled water, samples were dehydrated through 70, 80, 90 and 100% ethanol (5 min. in each stage) at room temperature. Ethanol was then replaced by liquid CO₂ and the samples were air dried (Llorca and Valiente, 1993). Then samples were mounted on stubs and

coated with gold. These coated specimens were observed at 15 kV in SEM (Hitachi, Japan).

Inhibition of spore germination

To investigate the inhibition of spore germination, Oak pyroligneous liquor was purchased which is available in the commercial market. 200 counted spores were taken from the pre-isolated culture and 0% to 50% concentration (v/v) of oak pyroligneous liquor, with 5% Dimethyl Sulfoxide (DMSO) together was used to check the percentage of the inhibition. Spore suspension of 200 spores was made, then different concentration of oak pyroligneous liquor with 5% DMSO was mixed in the suspension and pouring of the final suspension was done on the slide prepared of 2% water agar. Triplicate slides were prepared and kept it into Petri plates. Then the plates were incubated at 26°C for 56 hrs. After completion of the incubation period counting of the spore was done.

Gas chromatography-mass spectrometry (GC-MS)

To check the exact chemical composition, GC-MS of Oak pyroligneous liquor was carried out. The GC-MS analysis was performed using a Shimadzu GC-MS (GC-17A, Kyoto, Japan) equipped with a ZB-1 Ms fused silica capillary column (30m \times 0.25 mm i.d., film thickness 0.25 μ m). For GC-MS detection, electron ionization energy of 70 eV was used. Helium gas was used as the carrier gas at a constant flow rate of 1 ml/min. Injector and MS transfer line temperature were set at 220 and 290°C, respectively. The oven temperature was programmed 50 to 150°C at 3°C/min, then held isothermal for 10 min and finally raised to 250°C at 10°C/min. Diluted samples (1/100, v/v, in methanol) of 1 μ l were manually injected in the split less mode. The relative percentage of the oil constituents was expressed as percentage by peak area normalization. Identification of components of the Oak pyroligneous liquor was based on their retention indices, relative to a homologous series of n-alkane (C8-C20) on the ZB-1 capillary column under the same operating conditions and computer matching with the Wiley 6.0 libraries, as well as by comparison of the fragmentation patterns of the mass spectra with those reported in the literature data (Adams, 2001).

Results and Discussion

The results of relative composition with the name of the compounds those were identified by GC-MS analyses from

Table 1. Chemical composition of Oak pyriligneous liquor analyzed by GC-MS

RI ^a	Components	RA ^b	Identification ^c
674	Pyridine	2.18	RI, MS
676	Propanoic acid	5.32	RI, MS
724	Methyl glycol	1.91	RI, MS
787	Alpha-Methylpyridine	1.73	RI, MS
825	Butyrolactone	3.41	RI, MS
957	4-Methylenecyclohexanone	10.69	RI, MS
1003	3-Methy-1,2-cyclopentanedione	3.54	RI, MS
1090	o-Methoxyphenol	25.93	RI, MS
1148	Pantolactone	2.29	RI, MS
1204	Phenyl carbamate	4.23	RI, MS
1235	2,3-Dihydroxytoluene	7.84	RI, MS
1279	2,6-Dimethoxy phenol	16.06	RI, MS
1404	Levoglucosan	6.14	RI, MS
1470	Methyl vanillate	1.96	RI, MS
2498	Hexadecanoic acid	2.85	RI, MS
	Total	96.08	

^aRetention indices relative to *n*-alkanes C₈-C₂₀ on ZB-1 capillary column.

^bRelative area (peak area relative to the total peak area).

^cIdentification: MS, comparison of mass spectra with MS libraries; RI, comparison of retention index with bibliography.

the Oak pyriligneous liquor are presented in Table 1 according to their elution order on a ZB-1 capillary column. Upon GC-MS analysis, we found 15 different compounds, representing 96.08% of the total sample. The major compounds detected were *o*-methoxyphenol (25.93%), 2,6-dimethoxy phenol (16.06%), 4-methylenecyclohexanone (10.69%), 2,3-dihydroxytoluene (7.84%), levoglucosan (6.14%), propanoic acid (5.32%), and phenyl carbamate (4.23%). However, it is noteworthy that the chemical composition of the extract from a particular species of plant can differ between harvesting seasons, extraction methods, and geographical sources, and that those from the different parts of the same plant can also differ widely (Burt, 2004).

The rust group is considered as one of the most dangerous pathogens to agriculture and horticulture. All rusts are obligate parasites, meaning that they require a living host to complete their life cycle. They generally do not kill the host plant but can severely reduce growth and yield. Food and feed spoilage fungi cause great economic losses worldwide. It is estimated that between 5 and 10% of the world agricultural production is wasted due to fungal deterioration (Pitt and Hocking, 1997). Rust disease of *Perilla* is highly frequent and is widely spread in Korea (Al-Reza *et al.*,

2010). As this is very difficult to germinate the spore in vitro, we have emphasized our work on germination of the spore in water agar medium, to maintain the humidity; saturated salts were prepared and poured into Petri dishes that had been lined with five layers of tissue paper (Kimtech™) to soak up the saturated salt solution and to prevent spillage. Potassium chloride (KCl), potassium nitrate (KNO₃) and water were used to achieve

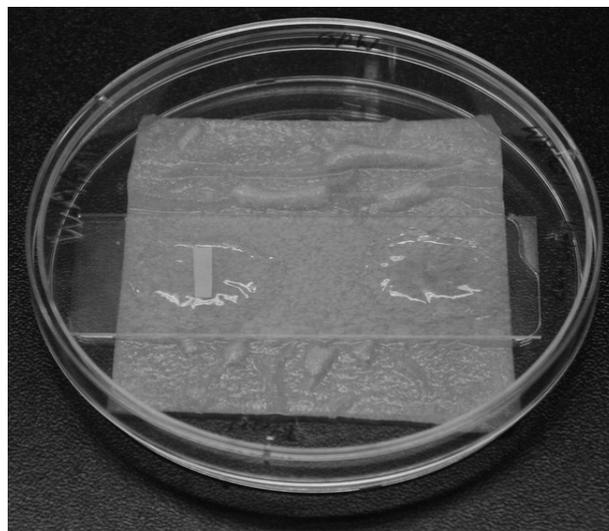


Fig. 1. In vitro assay for urediniospore germination of perilla rust fungi in water agar media.

Table 2. Effect of Oak pyroligneous liquor on urediniospore inhibition

Sample	Conc. (V/V) in 5% DMSO	Average numbers of non-germinated spores out of 200 spores	Inhibition (%)
Oak pyroligneous liquor	50	63.6±13.6	31.8±6.8
	45	51.6±4.4	25.8±2.2
	40	41.3±3.7	20.6±1.8
	35	38.0±3.0	19.0±1.5
	30	22.6±4.4	11.0±2.2
	25	14.6±2.6	7.3±1.3
	20	7.6±3.4	3.8±1.7
	15	7.3±1.3	3.6±0.7
	10	7.0±4.0	3.5±2.0
5	6.6±2.6	3.3±1.3	
Control	0	8.0±1.0	4.0±0.5

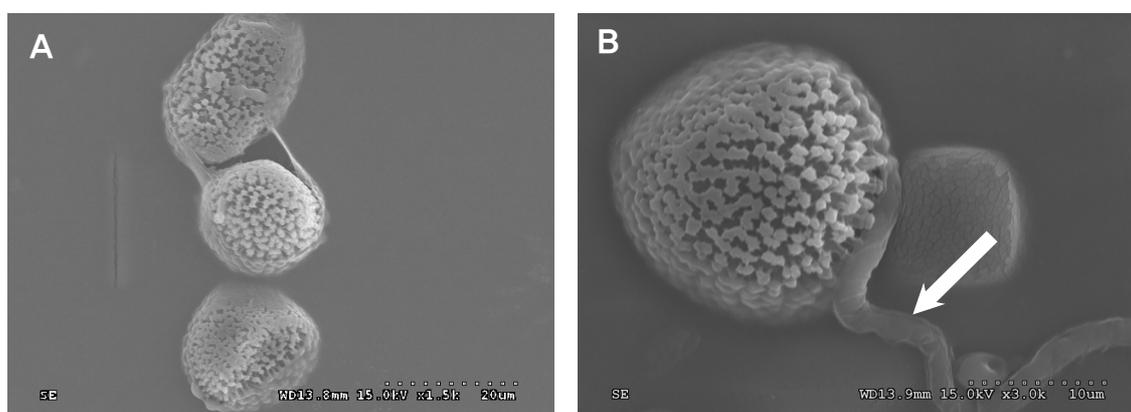


Fig. 2. Scanning electron microscopic view of *Perilla* rust *Coleosporium plectranthi*, urediniospore from the culture. Arrow in (B) is showing the germinated part. (A) non-germinated spore treated with Oak pyroligneous liquor; (B) germinated spore.

the proper humidity (Fig 1). A spore was considered as germinated when its germ tubes at least half the urediniospore length. We confirmed the germination of urediniospore by scanning electron microscope image which is shown in Fig 2. The effect of Oak pyroligneous liquor on the germination of spore is summarized in Table 2. The best inhibitory effect was recorded at the concentration of 50% (v/v) pyroligneous liquor compare to the others, as it has inhibited average of 63.6 spores out of 200. On the other hand, the lowest inhibition was found at the concentration of 5% (v/v) pyroligneous liquor sample as it was inhibited only 3.3% spores.

Thus, it can be concluded that the use of Oak pyroligneous liquor could be an alternative to synthetic fungicides for using in agro industries and also to screen and develop such novel types of selective and natural fungicides in the treatment of rust diseases of *Perilla* leaves causing severe destruction to *Perilla* plant cultivated in Korea.

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