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DEP: 2020 DIRECCION DE NUEVAS CREACIONES	EVE: 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS: 7
ACT: 519 PRESENTACION	

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
CARLOS REINALDO OLARTE GARCIA

Asunto: Radicación: 12-13958- -6
Trámite: 11
Evento: 378
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Oficio: 14593
Folios: 7

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	US2010/43612				
Fecha de Presentación Internacional	29/07/2010				

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	12-013958-00000-0000	30/enero/2012
Reivindicaciones:	12-013958-00000-0000	30/enero/2012
Dibujos:	12-013958-00000-0000	30/enero/2012
Prioridad:	US 61 230 102	30/julio/2009

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del Título X de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. En la presente solicitud se estudiaran las 15 reivindicaciones del radicada 12-013958-00000-0000 del 30 de enero de 2012.

Título de la solicitud: “Combinaciones de inhibidor de patógeno de planta y métodos de uso”

El problema planteado en esta solicitud consiste en la necesidad de modular la infección por parte de patógenos en plantas.

Para resolver este problema técnico la solicitud en estudio proporciona composiciones que comprenden extractos que contienen antraquinonas, un agente de control biológico o fitopatogénico, y un tensoactivo.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A 01 N 25/30; A 01 N 35/06; A 01 N 43/48; A 01 N 43/64; A 01 N 65/30

4. Reivindicaciones relativas a un uso o a un segundo uso (Art 14 y 21 D 486)

El uso de las reivindicaciones 8, 12 a 14 no es patentable, de acuerdo con el Art 14, ya que si bien dichas reivindicaciones se encuentran encabezadas como composiciones, las características proporcionadas se refieren al uso y no a los componentes de la composición.

5. De la solicitud de patente

Titulo

Se sugiere al solicitante modificar el título de la solicitud de manera que no haga referencia a usos, debido a que corresponde a materia no patentable bajo el Art. 14 y 21 D 486.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

El capítulo reivindicatorio no es claro porque no define la composición reclamada mediante la caracterización estructural de los componentes y sus proporciones. Específicamente la solicitud no proporciona:

- Los componentes y proporciones del extracto de planta, así como la identificación del compuesto(s) marcador(es) de dicho extracto, su proporción dentro del extracto y la planta a partir de la cual es obtenido dicho extracto, de manera que un experto en la materia sea capaz de obtener dicho extracto con la seguridad que presentará la actividad deseada.
- No define los posibles tensoactivos útiles para la realización de la composición.
- Si bien la solicitud presenta un listado para los posibles agentes anti-fitopatogénicos y agentes de control biológico, la descripción de la misma no sustenta el efecto sinérgico con cada uno de los agentes reclamados. Se requiere al solicitante que restrinja sus reclamaciones a las combinaciones que tienen pleno sustento de su efecto técnico en la descripción de la solicitud. La siguiente tabla muestra que combinaciones tienen sustento en la descripción:

Reivindicación	Sustento
Reivindicación 5 Combinación de: I. Extracto que contiene derivados de antraquinona II. Agente antifúngico a. De sitio único: Benzimidazol, morfolina, hidroxipirimidina, anilino pirimidina, fosforotiolato, inhibidor externo de quinona, quinolina, dicarboximida, carboximida, fenilamida, anilino pirimidina, fenilpirrol, hidrocarburo aromático, ácido cinámico, hidroxianilida,	Combinación de: I. Extracto que contiene derivados de antraquinona II. Fungicida químico no inorgánico multisitio: Mefenoxam (Pág. Fl. 66 Tabla 13)

antibiótico, polioxina, acilamina, ftalimida, bencenoide {xililalanina}; b. Un inhibidor de desmetilación seleccionado del grupo que consiste en iraidazol, piperazina, pirimidina, y triazol c. Un aceite o producto oleoso natural que tiene actividad fungicida y/o insecticida d. Un fungicida químico no inorgánico multisitio: nitrilo, cobre, quinoxalina, sulfamida, fosfonato, fosfito, ditiocarbarnato, cloralquiltios, fenilpiridin-amina, ciano-acetamida oxima/ fludioxoniloo y mefenoxam.	
Reivindicación 6 Combinación de: I. Extracto que contiene derivados de antroquinona II. Agente antimicrobiano es un a. Triazol seleccionado de bitertanol, miclobutanilo, penconazol, propiconazol, triadimefon, bromuconazol, ciproconazol, diniconazol, fenbuconazol, hexaconazol, tebuconazol, tetraconazol, b. Un imidazol, en donde dicho imidazol es fenamidona, fenapanilo, iprodiona, triflumizol c. Un nitrilo, en donde dicho nitrilo es cloronitrilo y fludioxonilo; d. Una estrobilurina; e. Quinona, en donde en donde dicha quinona es quinoxifeno (Eter 5,7-dicloro-4-quinolil-4-fluorofenilico).	Combinación de: I. Extracto que contiene derivados de antroquinona II. Agente antimicrobiano es un a. Triazol: Miclobutanilo (Fl 60 Tabla 1, Fl 62 Tabla 2), Propiconazol (Fl. 68 Tabla 19) b. Imidazol: Triflumizol (Fl. 65 Tabla 9) c. Nitrilo: Fludioxonilo (Fl. 67 Tabla 17) d. Quinona: Quinoxifeno (Fl. 62 Tablas 5 y 6),
Reivindicación 7: Combinación de: I. Extracto que contiene derivados de antroquinona II. Donde dicho agente antimicrobiano es estrobilurina en donde dicha estrobilurina es azoxistrobina, kresoxim-metilo o trifloxistrobina.	Combinación de: I. Extracto que contiene derivados de antroquinona II. Azoxistrobina (Fl. 68 Tabla 7)

Se aclara al solicitante que no se puede definir ningún elemento de la composición en términos únicamente funcionales como es el caso de caracterizar un componente simplemente por ser un agente fitopatogénico, la definición debe tener un carácter estructural.

Adicionalmente la solicitud no es clara puesto que contiene “disclaimers” que no tienen sustento en la descripción. Específicamente la solicitud hace referencia a la exclusión de de agentes de control biológico de origen *Bacillus*, *Pseudomonas*, *Brevabacillus*, *Lecanicillium*,

Ampelomyces, *Phoma*, *Pseudomona*, o que no sea vitamina E o un agente organofosforado, sin embargo dichos “disclaimers” no tienen sustento puesto que la descripción no proporciona argumentos para la exclusión de dichos agentes.

Descripción (Art 28 D 486)

La descripción de la solicitud no es clara puesto que contiene “disclaimers” que no tienen sustento, específicamente el solicitante no justifica la exclusión de agentes de control biológico de origen *Bacillus*, *Pseudomonas*, *Brevabacillus*, *Lecanicillium*, *Ampelomyces*, *Phoma*, *Pseudomona*, o que no sea vitamina E o un agente organofosforado, entendiendo que la justificación no se limita a la simple mención de las exclusiones, sino a la explicación del efecto técnico por el cual se excluyen.

Por otra parte, la descripción no provee ejemplos en los cuales el solicitante demuestre haber obtenido el extracto de planta, la caracterización del mismo y la medición de la actividad descrita, de manera que no es posible saber si el solicitante se encuentra en posesión de dicho extracto.

6. Unidad de invención (Art 25 D 486)

La solicitud no cumple con el requisito de unidad de invención porque se refiere 7 grupos inventivos para las combinaciones que tienen sustento en la descripción, dichos grupos inventivos están definidos por las siguientes características esenciales:

1. Reivindicaciones que reclaman la combinación de I. Extracto que contiene derivados de antroquinona y II: Agente antimicrobiano antroquinona
2. Reivindicaciones que reclaman la combinación de I. Extracto que contiene derivados de antroquinona y II: Agente antimicrobiano mefenoxam
3. Reivindicaciones que reclaman la combinación de I. Extracto que contiene derivados de antroquinona y II: Agente antimicrobiano Miclobutanilo
4. Reivindicaciones que reclaman la combinación de I. Extracto que contiene derivados de antroquinona y II: Agente antimicrobiano Triflumizol
5. Reivindicaciones que reclaman la combinación de I. Extracto que contiene derivados de antroquinona y II: Agente antimicrobiano Fludioxonilo
6. Reivindicaciones que reclaman la combinación de I. Extracto que contiene derivados de antroquinona y II: Agente antimicrobiano Quinoxifeno
7. Reivindicaciones que reclaman la combinación de I. Extracto que contiene derivados de antroquinona y II: Agente antimicrobiano Azoxistrobina.

No hay unidad de invención entre 7 grupos independientes ya que las composiciones contienen dos posibles grupos de compuestos: I. Extracto que contiene derivados de antroquinona y II: Agente antimicrobiano, sin embargo los agentes antimicrobianos reclamados comprenden estructuras completamente diferentes, por lo cual no pueden considerarse todas las combinaciones reclamadas como un solo concepto único inventivo.

Se le sugiere al solicitante restringir la materia a patentar en un solo concepto común inventivo especificando el avance tecnológico sobre el estado de la técnica, y teniendo en cuenta el sustento proporcionado en la descripción. Esto se logra entre otras formas definiendo las características esenciales de la invención y mostrando las otras características particulares como dependientes, pero sin perjuicio de la concisión de las nuevas reivindicaciones tanto independientes como dependientes.

El solicitante también puede realizar una o más de las divisionales de acuerdo a los grupos de conceptos técnicos inventivos presentados en esta solicitud, esto claro, sin ampliar el objeto inicial de la invención y teniendo en cuenta las combinaciones sustentadas en la descripción.

7. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: 30 de julio de 2009, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación*
D1	WO2006037632	Compositions fongicides synergiques	16/abril/2006
D2	Pest Manag Sci 63:511–515 (2007)	Synergistic interaction of physcion and chrysophanol on plant powdery mildew	2007

El documento D1¹ divulga composiciones fungicidas para el tratamiento de enfermedades fitopatogenicas en plantas.

El documento D2² divulga extractos de *Reynoutria* para control fitopatogenico y describe los efectos sinergicoss de las antraquinonas presentes en dicho extracto en la actividad anti-fitopatogénica (Pág. 511 resumen).

8. Examen de Patentabilidad (Art 14 D486)

Nivel inventivo (Art18 D486)

La reivindicación 1 consiste en “Una combinación que comprende: (a) un extracto que contiene uno o más derivados de antraquinona que inducen la resistencia de las plantas a los fitopatogenos y derivados de una planta y (b) uno o más agentes anti-fitopatogenicos seleccionados del grupo que consiste en: (i) un no benzotiazol, no vitamina E, agente antimicrobiano no organofosforado, que carece de azufre no elemental o no humectable (ii) un tensoactivo que tiene actividad fungicida, y/o (iii) un agente de control biológico no *Bacillus*, no *Pseudomonas*, no *Brevabacillus*, no *Lecanicillium*, no *Ampelomyces*, no *Phoma*, no *Pseudozyma*.”

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1	D2
Combinación	Composición con dos componentes (Pág 1 ln 2-3):	
Extracto que contiene uno o más derivados de antraquinona que inducen la resistencia de las plantas a los fitopatogenos y derivados de una planta	Extracto de <i>Reynoutria</i> y/o antraquinonas (Pág. 24 ln 18 y Pág. 25 ln 2)	Extracto de <i>Reynoutria</i> caracterizado por contener antraquinonas physcion y chrysophanol (resumen Pág. 511) que proveen resistencia a plantas.
Uno o más agentes anti-fitopatogenicos, o Un tensoactivo, o un agente de control biológico	Agente fungicida de formula I (Pág. 1)	

El documento D1 se considera el estado de la técnica cercano a la reivindicación 1 porque divulga una composición con dos componentes (Pág 1 ln 2-3) que comprende un extracto de *Reynoutria* y/o antraquinonas (Pág. 24 ln 18 y Pág. 25 ln 2) y un agente agente fungicida de formula I (Pág. 1), dicho documento divulga además un método para modular una infección fito patogénica que comprende aplicar una cantidad sinérgicamente efectiva de la composición descrita (Pág. 86 ln 11 a 14).

La diferencia entre la composición definida en la reivindicación 1 y la divulgada en el documento D1 consiste en que la solicitud hace referencia a extractos de plantas que presentan actividad anti-fitopatogenica, mientras que el documento D1 di bien menciona extractos de *Reynoutria* no menciona que dihcoss extractos tengan la actividad deseada.

El efecto que logra el incluir la inclusión de extractos de plantas que contienen antraquinonas es que presentan un efecto sinérgico en su combinación con otros agentes fitopatogenicos (Pág. 1 ln 13)

1URL:http://worldwide.espacenet.com/publicationDetails/description?CC=WO&NR=2006037632A1&KC=A1&FT=D&ND=3&date=20060413&DB=EPODOC&locale=en_EP
2URL <http://www.ncbi.nlm.nih.gov/pubmed/17397111>

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: cómo diseñar composiciones alternativas con actividad anti-fitopatogenica.

Sin embargo, la utilización de extractos de *Reynoutria* para control fitopatogenico ya se encuentra divulgado en el documento D2, que describe los efectos sinergicoss de las antraquinonas presentes en dicho extracto en la actividad anti-fitopatogenica (Pág. 511 resumen), y el documento D1 sugiere el uso de antraquinonas provenientes de la misma planta con el mismo efecto técnico divulgado en la presente solicitud (Pág. 24 ln 18 y Pág. 25 ln 2).

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría de extractos de *Reynoutria* del documento D2 en la composición divulgada en el documento D1 para llegar así al objeto de la reivindicación 1 de la solicitud, por lo cual se considera obvia.

Las reivindicaciones 2 a 11 y 15 no presentan características técnicas adicionales por lo cual se considera que tampoco cumplen con el requisito de nivel inventivo.

9. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	WO2006037632	1 a 11 y 15	Nivel inventivo
D2	Pest Manag Sci 63:511–515 (2007)	1 a 11 y 15	Nivel inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2013-08-13

Elaboró: Sandra Carolina Crespo
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Synergistic interaction of physcion and chrysophanol on plant powdery mildew

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Abstract: The extract of the plant *Rheum officinale* Baill, mainly containing the anthraquinones physcion and chrysophanol, is highly active against plant powdery mildew. Experiments were conducted in the laboratory and greenhouse to determine the interaction of the two compounds on cucumber powdery mildew [*Sphaerotheca fuliginea* (Schlecht.) Poll] and on wheat powdery mildew [*Blumeria graminis* (DC.) Speer f. sp. *tritici* Marchal]. Physcion was much more bioactive than chrysophanol against these powdery mildews. There was a significant synergistic interaction between the two compounds on the diseases when the ratios of physcion to chrysophanol ranged from 1:9 to 5:5. The synergistic degree increased with increase in the chrysophanol proportion in the combination. The findings indicate that, in order to ensure constant efficacy of the extract on the disease, both the contents and the proportion of the main active ingredients physcion and chrysophanol have to be determined.

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Keywords: anthraquinone; physcion; chrysophanol; bioassay; biopesticide; synergism; *Rheum officinale*

1 INTRODUCTION

Powdery mildew, caused by *Sphaerotheca fuliginea* (Schlecht.) Poll on cucumber and by *Blumeria graminis* (DC.) Speer f. sp. *tritici* Marchal on wheat, is a widespread disease in greenhouse and field crops and can bring about severe yield loss.^{1–3} Applying synthetic fungicides to control these diseases has resulted in environmental pollution and decrease in the sensitivity of plant pathogen populations to fungicides.⁴ To reduce the use of synthetic fungicides, an alternative measure is the use of plant extracts to control the disease.^{5–7}

Physcion and chrysophanol are natural anthraquinone derivatives found in three plant families: Fabaceae (*Cassia* spp.), Polygonaceae (*Rheum*, *Rumex* and *Polygonum* spp.) and Rhamnaceae (*Rhamnus* and *Ventilago* spp.). They can be isolated from the roots of the plants by extraction and column chromatography.^{5,8–10} They are the major active ingredients in traditional herbal medicines widely used in clinics^{11,12} and have numerous types of biological activity.^{13–17} Recent studies have indicated that these anthraquinones can affect the immune system, vasomotor system and metabolic processes. Other biological activities include anti-inflammatory,¹⁸ antitumour,¹⁹ antioxidant,²⁰ antifeedant²¹ and antimicro-organism.^{22–23}

Physcion and chrysophanol also have activity against phytopathogens, such as barley powdery mildew (*B. graminis* f. sp. *hordei*), cucumber powdery mildew (*S. fuliginea*), rice sheath blight (*Rhizoctonia solani* Kuhn), grey mould (*Botrytis cinerea* Pers ex Pers) and

cucumber downy mildew [*Pseudoperonospora cubensis* de Bary].^{5,24} During a study on their efficacy against wheat powdery mildew, the efficacy of natural extracts mainly containing physcion and chrysophanol was found to be significantly higher than that of the individual compounds at the same dosage, indicating potential synergism between them. To confirm this phenomenon, a series of studies was conducted in the laboratory and greenhouse.

2 MATERIALS AND METHODS

2.1 Compounds

Physcion (98%) and chrysophanol (98%) (Fig. 1) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China.

The natural extract used in the greenhouse experiments was obtained by a modification of Chen's method.²⁵ Ground rhizomes of *R. officinale*, belonging to the family of Polygonaceae, purchased from Beijing Tongrentang Co., Ltd, were refluxed with a solution of 20% aqueous sulfuric acid + chloroform (1 + 5 by volume) in a water bath for 3 h. The chloroform phase was extracted with 2.5 g L⁻¹ aqueous potassium hydroxide, and the organic solvent layer was then extracted with 50 g L⁻¹ aqueous potassium hydroxide; this second aqueous fraction was acidified with hydrochloric acid to yield a yellow precipitate. The precipitate was filtered, washed with distilled water and dried, yielding a mixture of chrysophanol and physcion. Their RF values on a chromatoplate (silica gel 200 mesh,

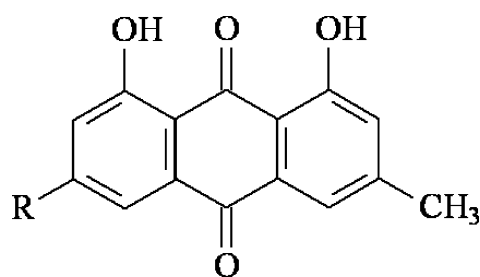
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Physcion R = OCH₃

Chrysophanol R = H

Figure 1. Structures of the compounds tested.

0.25 mm) were 0.74 and 0.53 respectively, using petroleum ether + ethyl acetate (15 + 1 by volume) as eluent. The ratio of physcion to chrysophanol in the extract was 22:78 based on a GC analysis using a gas chromatograph with a flame ionization detector (Agilent 6890 N/5973 Inert MSD) and a column of DB-5MS (30 m × 0.25 mm ID × 0.25 µm). The carrier gas was helium at a flowrate of 2 mL min⁻¹. The oven temperature was adjusted as follows: initial temperature 95 °C held for 2 min, raised to 230 °C at 25 °C min⁻¹ and held for 5 min at 230 °C and then raised continuously to 290 °C at 20 °C min⁻¹ and held for 15 min at 290 °C. Both the injector and detector temperatures were set at 250 °C. The injected volume was 2 µL. The retention times of chrysophanol and physcion were 18.95 and 23.50 min respectively.

Natural extract, physcion and chrysophanol were separately dissolved in acetone (1.0 mg mL⁻¹) as stock solutions before application.

2.2 Preparation of conidium inoculum

Isolates of *S. fuliginea* and *B. graminis* were provided by the Plant Pathology Lab of the Institute for Plant Protection and Soil Sciences, Hubei Academy of Agricultural Sciences, China, and reproduced on cucumber and wheat plants respectively. Cucumber powdery mildew was reproduced in a growth chamber at 25 ± 1 °C and 70% relative humidity with a 16:8 h light:dark photoperiod. The spores of wheat powdery mildew were reproduced on wheat in a growth chamber at 18 ± 1 °C and constant light. Once adequate amounts of spores had been produced for the experiment, the leaves of cucumber were rinsed with sterilized distilled water to prepare the inoculation suspension of *S. fuliginea* (about 2 × 10⁵ spores mL⁻¹). Conidia on wheat leaves were used directly for inoculation.

2.3 Laboratory experiment

The obligate biotroph fungi *S. fuliginea* and *B. graminis* cannot be cultured *in vitro*, so the determination of bioactivity of fungicides on wheat powdery mildew and cucumber powdery mildew was conducted *in vivo* on leaf segments for the former disease and on leaf discs for the latter.

Seeds of cucumber cultivar Changchunmici, susceptible to cucumber powdery mildew, were sown in 200 mm diameter plastic pots in a glasshouse. About 3–4 weeks later, the fourth leaves of seedlings were detached, washed with distilled water, dried on a paper towel and then cut into 15 mm discs. Six leaf discs were placed in a 90 mm diameter petri dish containing 20 mL water agar. Seeds of wheat cultivar Zheng 98, susceptible to wheat powdery mildew, were sown in 200 mm diameter plastic pots in a growth chamber at 18 ± 0.5 °C and 70% RH and with constant light of 72 µmol m⁻² s⁻¹. The leaves of ten-day-old seedlings were cut into 40 mm segments and then placed in petri dishes (five per dish) with water agar.

Physcion and the nine combinations of physcion and chrysophanol (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8 and 1:9, w/w) were applied at rates of 10, 2.5, 0.63, 0.15 and 0.04 µg mL⁻¹, and chrysophanol was applied at 40, 20, 10, 2.5 and 0.63 µg mL⁻¹. Tween 80 was added in the final dilutions at a rate of 0.25 mL L⁻¹. Spraying was done at 0.25 MPa by means of an autospraying tower (Model PWT-510, manufactured by the Pesticide Machinery Group of the China Agricultural University) using a spray volume of 25 mL m⁻². In the preparatory experiments, there was no difference between untreated and solvent-treated controls in disease severity, and so a solvent-treated control was used in the laboratory experiment.

Cucumber leaf discs were inoculated by pipette with an *S. fuliginea* conidium suspension (5 µL) after application. The petri dishes were covered immediately after inoculation and placed in a moist chamber in the dark at 25 ± 1 °C overnight, and then moved into a growth chamber with a 16:8 h light:dark photoperiod for disease development. The wheat leaf segments were inoculated by spraying at a concentration of 2–4 × 10⁵ spores mL⁻¹ (counted with a hemocytometer), and then incubated in the growth room at 18 ± 1 °C and constant light.

Disease severities were scored 7 days after inoculation as percentage leaf area with powdery mildew, and converted to percentage disease control according to Abbott's method:²⁶

$$\text{Disease control (\%)} = 100 \times [(\text{untreated} - \text{treated}) / \text{untreated}]$$

2.4 Greenhouse experiment

To confirm further the synergism between physcion and chrysophanol in the natural extract, both physcion and chrysophanol at 10 µg mL⁻¹ and the natural extract at 10, 2.5 and 0.63 µg mL⁻¹ were applied to run off using a hand sprayer. Solvent-treated plants were used as controls. Inoculation was done 24 h after application.

Seedlings of wheat, cultivar Zheng 98, were grown in 200 mm pots, 14 plants a pot, in a greenhouse at ambient temperature (about 14–22 °C). When the second leaves were fully expanded, the plants were inoculated by blowing spores of wheat powdery

mildew onto the leaves with a rubber syringe in the late afternoon. The control plants had almost reached disease severity greater than 50% infection 15 days after inoculation. The disease severity was scored once according to the following scale: 0 indicated no disease; 1 indicated less than 5% cover of leaves by mildew; 3 indicated 6–15% cover; 5 indicated 16–25% cover; 7 indicated 26–50% cover; 9 indicated more than 51% cover of leaves by mildew.

Seedlings of cucumber, cultivar Changchunmici, were grown in 400 mm pots, five plants a pot, in a greenhouse at 18–25 °C. At the 4–5 true leaf stage, a conidium suspension of cucumber powdery mildew containing 2×10^5 spores mL⁻¹ was sprayed onto the leaves to run off by a hand sprayer in the late afternoon. The control efficacy was assessed 14 days after treatment by counting the number of colonies on the leaves.³ Both laboratory and greenhouse experiments were randomized designs with four replicates, and were repeated once.

2.5 Statistics and assessment of synergism

Dose–response regression was done by SSPS 10.0. The EC₅₀ values of physcion, chrysophanol and their combinations were calculated according to the method of Gisi *et al.*,²⁷ the theoretical EC₅₀ (EC_{50(th)}) being given by

$$EC_{50(th)} = (a + b)/(a/EC_{50(A)} + b/EC_{50(B)})$$

where A and B represent physcion and chrysophanol respectively, and *a* and *b* are the ratios of the two compounds in the combinations.

The type of interaction between physcion and chrysophanol was determined according to the following ratio:

$$R = EC_{50(th)}/EC_{50(ob)}$$

where EC_{50(ob)} is the EC₅₀ value calculated according to observed data. An *R* value not significantly different from 1.0 (between 0.5 and 1.5) indicates additive

response, an *R* value not less than 1.5 indicates a synergistic response and an *R* value less than 0.5 indicates an antagonistic response.²⁷

For the greenhouse experiment, data were subjected to analysis of variance. The comparison of treatment means was done using Duncan's multiple range test at the *P* = 0.05 level.

3 RESULTS

3.1 Laboratory experiment

Similar results were obtained from the two repeated experiments on wheat and cucumber powdery mildew (Tables 1 and 2). Physcion was more active than chrysophanol against both mildew diseases. The *R* value (theoretical EC₅₀/observed EC₅₀) of the combinations of the two compounds ranged from 0.99 to 2.78 for wheat powdery mildew and from 1.01 to 4.06 for cucumber powdery mildew. Based on Gisi's standard,²⁷ there was an additive effect when the combinations of physcion and chrysophanol were at a ratio of 6:4–9:1 and a synergistic effect when the combinations of the two compounds were at a ratio of 1:9–5:5 on both diseases. The synergistic level increased with increase in the chrysophanol proportion in the combination.

3.2 Greenhouse experiment

After being treated with the natural extract at 0.63–10 µg mL⁻¹, the disease severity on wheat seedlings and the colony number on cucumber leaves were significantly lower than on those treated with chrysophanol at 10 µg mL⁻¹. There were no differences in the disease severity and the colony number between the natural extract and physcion alone at the same concentration (Table 3). This showed that physcion and the natural extract had much higher activity on powdery mildew than chrysophanol. The natural extract contained a relatively low amount of physcion and could provide similar control of powdery mildew to physcion applied

Table 1. Fungicidal activities *in vivo* of physcion, chrysophanol and their combinations on wheat powdery mildew in the laboratory

Fungicide ^a	First test EC ₅₀ (µg mL ⁻¹)		<i>R</i> ^b	Second test EC ₅₀ (µg mL ⁻¹)		<i>R</i> ^b
	Theoretical	Observed(95% CL)		Theoretical	Observed (95% CL)	
Physcion	–	0.14 (0.12–0.16)	–	–	0.15 (0.13–0.17)	–
Chrysophanol	–	5.63 (4.09–7.17)	–	–	5.19 (4.40–5.98)	–
Combination 9:1	0.16	0.16 (0.14–0.18)	0.99	0.16	0.16 (0.14–0.19)	1.00
Combination 8:2	0.17	0.14 (0.13–0.16)	1.23	0.18	0.18 (0.15–0.20)	1.05
Combination 3:7	0.20	0.17 (0.14–0.20)	1.19	0.21	0.19 (0.16–0.21)	1.12
Combination 4:6	0.23	0.18 (0.15–0.22)	1.26	0.24	0.19 (0.17–0.22)	1.25
Combination 5:5	0.27	0.18 (0.16–0.21)	1.49	0.29	0.19 (0.16–0.21)	1.53
Combination 4:6	0.34	0.20 (0.17–0.24)	1.67	0.36	0.22 (0.19–0.26)	1.58
Combination 3:7	0.44	0.24 (0.21–0.26)	1.88	0.46	0.25 (0.20–0.29)	1.89
Combination 2:8	0.64	0.24 (0.20–0.28)	2.70	0.66	0.25 (0.22–0.29)	2.63
Combination 1:9	1.15	0.41 (0.37–0.46)	2.78	1.18	0.44 (0.38–0.49)	2.71

^a Ratio of combinations is physcion to chrysophanol.

^b Ratio of theoretical EC₅₀ to observed EC₅₀.

Table 2. Fungicidal activities *in vivo* of physcion, chrysophanol and their combinations on cucumber powdery mildew in the laboratory

Fungicide ^a	First test EC ₅₀ (μg mL ⁻¹)		R ^b	Second test EC ₅₀ (μg mL ⁻¹)		R ^b
	Theoretical	Observed (95% CL)		Theoretical	Observed (95% CL)	
Physcion	–	0.23 (0.21–0.26)	–	–	0.24 (0.21–0.27)	–
Chrysophanol	–	6.47 (5.55–7.34)	–	–	7.05 (6.18–7.93)	–
Combination 9:1	0.26	0.25 (0.22–0.28)	1.01	0.27	0.24 (0.21–0.27)	1.11
Combination 8:2	0.29	0.26 (0.22–0.29)	1.13	0.30	0.25 (0.22–0.28)	1.18
Combination 3:7	0.33	0.27 (0.24–0.30)	1.21	0.34	0.27 (0.23–0.30)	1.27
Combination 4:6	0.38	0.28 (0.24–0.31)	1.37	0.39	0.30 (0.26–0.33)	1.32
Combination 5:5	0.45	0.28 (0.25–0.31)	1.60	0.46	0.30 (0.26–0.33)	1.57
Combination 4:6	0.55	0.28 (0.25–0.32)	1.93	0.57	0.30 (0.26–0.34)	1.90
Combination 3:7	0.71	0.39 (0.35–0.44)	1.82	0.74	0.42 (0.37–0.47)	1.78
Combination 2:8	1.01	0.39 (0.35–0.44)	2.57	1.05	0.41 (0.35–0.46)	2.60
Combination 1:9	1.75	0.47 (0.41–0.53)	3.72	1.83	0.45 (0.40–0.50)	4.06

^a Ratio of combinations is physcion to chrysophanol.^b Ratio of theoretical EC₅₀ to observed EC₅₀.**Table 3.** Effects of physcion, chrysophanol and their natural extract on plant powdery mildew in greenhouse^a

Treatment (μg mL ⁻¹)		Disease index on wheat		Disease colonies on cucumber (no/plant)	
		First test	Second test	First test	Second test
Control	–	76.6 a	74.0 a	128.4 a	115.2 a
Physcion	10.00	4.9 e	4.4 e	7.7 d	6.7 e
Chrysophanol	10.00	66.3 b	61.1 b	113.8 b	99.9 b
Natural extract ^b	10.00	6.3 e	5.0 e	8.1 d	7.8 de
	2.50	10.7 d	9.4 d	13.7 d	10.5 d
	0.63	19.0 c	18.1 c	26.8 c	22.9 c

^a Means within a column followed by the same letter are not significantly different at $P = 0.05$.^b Ratio of physcion to chrysophanol is 22:78 (w/w).

alone. This confirmed that synergism existed between physcion and chrysophanol.

4 DISCUSSION

Traditional herbal medicines usually contain several active ingredients with synergistic properties. A combination of anthraquinones in phytomedicines has shown synergism to create a higher laxative effect in the human body.⁹ The anthraquinone derivatives physcion and chrysophanol are the active ingredients in the traditional medical herb *R. officinale* and are also effective at controlling plant diseases.^{5–7} The findings of this study showed the potential synergism between the two compounds in controlling plant powdery mildew.

Previous studies by the present authors found that the efficacy of *R. officinale* extracts mainly containing physcion and chrysophanol against plant powdery mildew varied from batch to batch (data not presented). The present findings indicate that the variation could be due to the different proportions of the two compounds in the extracts of different batches. Different proportions of the two compounds had different degrees of synergism, resulting in different control efficacy.

Interactions may also exist among different components of other plant extracts as biofungicides. Therefore, when a natural extract is directly developed as a biopesticide, both the contents and the proportions of the main components need to be determined to ensure constant efficacy of the product.

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