Alternaria gansuense, a plant systematic fungal pathogen producing Swainsonine in vivo and in vitro

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Research Article

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Abstract

Astragalus adsurgens grows wildly in Eurasia and North America. The plant is considered a locoweed in the United States of America (USA) and Australia, and as forage in China. However, *Alternaria gansuense* (synonym: *Embellisia astragali*) systematically infects *A. adsurgens*, producing swainsonine(SW), which poisons domesticated animals with severe effects on the body weights, abdominal organs and blood enzymes of rats. We speculated that the *A. gansuense* SW-producing fungus could be morphologically and molecularly related to the locoweed endophyte, *Alternaria oxytropis*, which systematically grows in host plants. Therefore, pure cultures of the fungi from diseased plants or endophytic interaction collected from fields were assayed for SW using high-performance liquid chromatography linked to mass spectroscopy(HPLC-MS). The presence of SW was also detected in the *A. adsurgens, A. oxytropis* and diseased plants using β-ketoacyl synthase(KS) gene required for the SW synthesis. The contents of SW in mycelia of *A. gansuense* were 561.61 μg/g and ranged between 73.93 and 89.11 μg/g in diseased *A. adsurgens* plants. The contents of SW were 703.99 μg/g in mycelia of *A. oxytropis* isolated from locoweed plants *O. ochrocephala* and between 74.36 and 101.13 μg/g in *O. ochrocephala* with endophytes. However, the concentrations of SW found in healthy *A. adsurgens* plants were low and ranged from 7.75–18.62 μg/g, with a slight increase (15.55–32.37 μg/g) in those found in *O. ochrocephala* without endophytes. This study proves that *A. adsurgens* infected with *A. gansuense* are not safe for livestock and points to the need for caution when using the plants as feed.

Introduction

Astragalus adsurgens (Pall) is a perennial legume widely grown in Russia, Mongolia, Japan, Korea, and in the temperate geographical zones of North America (Sun et al. 1999). The plant was trained into a cultivated variety and widely grown in arid areas of northern China as forage named ‘standing milk-vetch’. It grows in harsh environments as a bee plant, a windbreaker, a cover plant to protect the soil from erosion and as green manure for crop rotation (Wang et al. 2015).

Despite its enormous importance, the fungus *Embellisia astragali*, recently described as *Alternaria gansuense*, has been found to cause yellow stunt and root rot (YSRR) disease in standing milk-vetch (Li 2007; Liu et al. 2016). The disease was first discovered in China in 2007 and is mainly distributed in Huanxian, Gansu, Yanchi, Ningxia, Hengshan, Shanxi, Ohan, InnerMongolia, Songshan, Jianping and Liaoning provinces of China (Li Yan Zhong et al. 2011; Zeng 2016). Genetic evolution analysis based on *gpd*, *rpb*2 and *tef-1* genes and phylogenetic analysis classified the pathogenic fungus A. gansuense in the wave bud spore group (Section Undifilium) *Alternaria gansuense* also has a close relationship with the endophytic fungus isolated from locoweed (Li Y Z et al. 2011; Liu et al. 2016; Woudenberg et al. 2013).

Alternaria gansuense systematically infects *A. adsurgens*, producing swainsonine (SW), which poisons animals causing severe effects on their growth and development. The producing of SW is also related to the endophytic symbionts. Some studies have shown that SW can cause animals a disease called

locoism and though plants containing SW below 0.004% are safe for sheep, low doses may lead to weight loss and biochemical lesions, while high doses may cause chronic disease in livestock after grazing on *A. adsurgens* or *Oxytropis* plants that contain SW for an extended period of several weeks (James et al. 1981; Stegelmeier et al. 1999). Generally, *Astragalus* species have higher concentrations of SW than species found in Oxytropis (Ralphs et al. 2008). Several studies also show that β-ketoacyl synthase (KS) and yeast amino acid oxidase genescan regulate the synthesis of SW (Cook et al. 2017).

Swainsonine, an indolizidine alkaloid known as glycosidase inhibitor, was first isolated from the plant Swainsona canescens (Cook et al. 2017; Elbein et al. 1981; Molyneux and James 1991). Swainsonine causes a phenocopy based on lysosomal shortage disease when administered to animals (Dorling et al. 1978; Dorling et al. 1980). The alkaloid is also found in many different plants that cause an irreversible lysosomal storage disease, known as locoism in the Western and the North of America, Canada and China (Molyneux and James 1982; Ralphs et al. 2008). Swainsonine damages and changes the synthesis of glycoproteins in cells, eventually leading to cell death (Elbein et al. 1981; Hamaguchi et al. 2007). After feeding, grazing animals receive chronic neurological diseases characterized by madness, depression, infertility, miscarriage and growth defects, neurological changes, reproductive disturbances, and emaciation (Cook et al. 2014). However, SW can also be used as medicine to contribute to human health (Hamaguchi et al. 2007; Santos et al. 2011). For instance, SW inhibits cancer cells' growth and metastasis and enhances human immunity (Hino et al. 1985; Lu et al. 2012). Depending on SW own properties and genes, or inhibition of glycoproteins, at domestic and abroad, SW can be detected and studied using quantitative real-time polymerase chain reaction (qPCR) (Cook et al. 2009a; Zhang et al. 2014), enzyme-linked immunosorbent assay (Smith et al. 1992; Wang et al. 2012), and chromatographic tools such as gas chromatography (GS) (Chen 2004; Cui et al. 2008), thin layer chromatography (Cook et al. 2017; Yu 2006), and a high-performance liquid chromatography-coupled with or without a mass spectrometer (HPLC-MS) (Gardner et al. 2001; Lu 2007).

Because of the extensive geographical distribution of *A. adsurgens*, the plant is more devastating and several other species of *Astragalus* may also be toxic to livestock and wildlife (Cook et al. 2009b; Cook et al. 2011; James 1972; Williams and James 1975). For example, after feeding on *A. adsurgens* infected with *A. gansuense* three weeks earlier, the liver of the mice become significantly enlarged, severely impairing its function, while alanine aminotransferase and lactate dehydrogenation increase dramatically in the blood (Li 2007). The whole-genome sequencing of *A. gansuense* suggests that it has five enzymes, which include yeast-sine dehydrogenase (SDH), polyketide synthase (PKS) associated with SW, and 170 genes annotated to the five enzymes (Xu 2017). In addition, molecular biology techniques have allowed the detection and characterization of SW in *Astragalus membranaceus* and its fungi directly (Li 2018). Therefore, the purpose of this research was to detect whether the diseased *A. adsurgens* and the pure cultures of A. gansuense had SW and whether plants infected by *A. gansuense* could cause poisoning in livestock.

Material And Methods

Plants Collection

Astragalus adsurgens were planted at Heping town in Lanzhou (103.58 E, 36.00 N) with an elevation of 1750m above sea level (m.a.s.l) in Gansu Province in 2010 (Zeng 2016). Lanzhou has a semi-arid continental climate, significant temperature variations and less rainfall, with an average annual precipitation of 300 to 400 mm. The incidence of YSRR in Lanzhou was 100% in 2015 (Zeng 2016). The incidence of YSRR disease occurred on 80% plants of *A. adsurgens* and twenty symptomatic and asymptomatic *A. adsurgens* plants were collected at the end of September 2017 and 2018, depending on its symptoms described in 2007 (Li 2007). Plants of *Oxytropis ochrocephala* used in the study were collected at the end of August 2017 from Haiyuan County, Ningxia Province (105.36° E, 36.29° N), with an elevation of 2173 m.a.s.l.

Fungi Materials

Pathogenic fungus was the isolate (MHLZU0408) preserved at the Mycological Herbarium of Lanzhou University (MHLZU) (Li and Nan 2007), cultured on wheat hay decoction agar (WHDA) (Li 2007). Endophytes was the strain isolated by Liu (2019) preserved at the Mycological Herbarium of Lanzhou University (MHLZU) (Liu 2019), cultured on potato dextrose agar(PDA) (Li 2007). These fungi had identified by using morphological and molecular characteristics (Li and Nan 2007; Yu et al. 2018). The pure cultures of fungi were collected and then dried in a freeze dryer for 24 h and stored in a refrigerator at -20°C before further experiments.

Extraction Of Dna

DNA from healthy and diseased *A. adsurgens* stems and leaves (five each), *O. ochrocephala* plants with and without endophytes (two each) were extracted using Plant DNA Kit (OMEGA Biotech Co.Ltd.) following the manufacturer's instructions from 10 mg of dried samples previously grounded to powder using sterile pestle. For fungus DNA extraction, the mycelia of pure cultures of *A. gansuense* and *A .oxytropis* were scraped from one fresh colony into a 2mL centrifugetube using a sterilized spoon. Genomic DNA was extracted using HP Fungal DNA Kit (OMEGA Biotech Co. Ltd.) following the manufacturer's instructions.

Polymerase Chain Reaction (PCR) Amplification

The KS gene was amplified using the specific primer pair KS-F(GAGGAAATTGCTATAGTTTCCATGGC) /KS-R (GGCATCCGAAAGACGTTTAAGAAG) (Cook et al. 2017). The PCR amplifications were performed in a 2720 thermal cycler (Applied Biosystems) in a total reaction volume of 25μL mixture containing 1μLof genomic DNA,1μLof forward and reverse primers(10μM), 12.5 μL of 2×Es TaqMasterMix (Dye) and 9.5 μL of sterile water as follows: an initial denaturation step was performed at 94°C for 5 min; followed by 29 cycles of denaturation at 94°C for 30 s, then annealing at 56°C for 30 s and extension at 72°C for 60 s;

and a final elongation step at 72°C for 4 min. Amplicons were visualized by gel electrophoresis in a 1% agarose gel stained with BBI 4S GelRed using 1X TAE (40 mM of Tris-acetate and 1 mM of EDTA at pH 8.0, purchased from Sangon Biotech) as a running buffer and photographed (Li 2018).

Assaying The Content Of Swainsonine

One-month-old fungal mycelia were scraped from one fresh colony using a sterile spoon and then dried in a Freeze dryer. About 100 mg of dried and ground stems were then placed into new 2 mL centrifuge tubes. The samples were freeze-dried for 24 h and then wrapped in labeled and sealed filter papers (10 cm × 4 cm). Each sample was continuously extracted with 60 mL methanol at 70°C in a water bath for 12 h using a Soxhlet apparatus extractor. A dry methanol extract was then carried out in a steam spinner to separate the plant material from the solvent layers. The final residue was dissolved in a 10 mL acetic acid solution (2%) in an ultrasonic cleaning machine for 30 min at 60°C. The solution was added to the cation-exchange extraction resin (732) adsorption column (6 cm) in which a small amount of cotton was placed into the tip, and a rubber septum was used to plug the tip end with the dynamic exchange for 2h. The resin and solution were then mixed for 30 min using mechanical rotation to allow the SW cations present in the sample to bind to the resin. The adsorption column was eluted with 50 mL of deionized water, which was then discarded, while for there incolumn, 50 mL ammonia solution (1 mol/L) was used. The ammonia eluate was dried in an evaporating dish and dissolved in 1 mL methanol. The sample solutions were placed into 2 mL centrifuge tubes, centrifuged at 12,000 rpm for 10 min at room temperature, and stored at -20°C until analysis for SW by HPLC-MS [38, 48]. All chemicals or solvents used were of analytical reagent grades. Acetic acid was 2% glacial, while water was deionized. The SW standard was purchased from Sigma USA.

To determine SW concentration by HPLC-MS, a stock solution of SW standards of 15.625, 156.25, 312.5,625, 937.5, and 1250μg/mL using serial dilution was prepared with methanol. After which, the samples and calibration standards were loaded into the autosample rvial holder for analysis (Wang et al. 2012).

The SW peak area was measured from the reconstructed ion chromatogram (m/z = 156) and quantitation based on an external calibration standard. The mobile phase consisted of acetonitrile,40% deionized water, and 60% methanol with 1% formic acid, MH = 174.2 ± 0.75amu, flow rate 0.5 mL/min, chromatographic column: Bestasil C18 (100×2mm, Keystone Scientific), injection volume1uL, capillary temperature 200°C, voltage:16V, peak time: 1.14min, wavelength: 205nm, detection mode: SIM mode, full MSScan = 174.11, using an HPLC-MS to determine the peak area of each concentration as the vertical coordinate and the standard solution concentration served as the horizontal ordinate (Gardner et al. 2001).

Data Analysis

Data were analyzed using IBM SPSS Statistics ver. 21.0 (SPSS, Inc., Chicago, USA). Swainsonine concentrations in *A. adsurgens* and locoweed stems and leaves and fungus were analyzed by one-way ANOVA to test differences. Tukey's HSD test was used to determine the significant differences between mean values at $P < 0.05$.

Results

Standard Curve

The regression equation y = 2486.81 x -13437 (R2 = 0.9981), obtained using the standard curve based on the prepared standards, was used to estimate the concentrations of SW in healthy and diseased *A. adsurgens* and *O. ochrocephala* plants with and without endophytic fungus. Peak emergence time was between 1 and 2 min and was neat and single without the phenomenon of towing (Fig. 1). The target ion was obtained in a mass spectrogram of 156.08 by crushing it with the energy of @cid from 25 to a total of 174.11 (Fig. 2).

Swainsonine In Plants

In the fields, the stems and leaves of the healthy-looking *A. adsurgens* plants were green, thick and well-nourished (Fig. 3A). However, the plants of *A. adsurgens* infected with *A. gansuense* were smaller, with several tillers or dwarfed branches; the average disease incidence was 80%. These infected plants were also characterized by brown petioles and discoloration of the stems from brown to dark brown (Fig. 3B). For *O. ochrocephala*, the endophyte was isolated from plants showed head sprouting with several matured seeds (Fig. 3C).

Generally, the contents of SW in healthy *A. adsurgens* stems were lower than those found in diseased stems and ranged from7.75-18.62 μg/g in healthy *A. adsurgens* stems and from 42.59-108.32 μg/g in diseased *A. adsurgens* stems. The study showed that the contents of SW detected in diseased *A. adsurgens* plants in 2017 and 2018 were significantly higher than those in healthy plants ($P < 0.05$; Fig. 4). Furthermore, the content of SW in *O. ochrocephala* with *A. oxytropis* was considerably more than in plants without endophytes ($P < 0.05$; Fig. 4). The contents of SW ranged from 74.36-101.13μg/g in endophytic *O. ochrocephala* plants and 15.55–32.37μg/g in plants without endophytes.

The colony of A. oxytropis isolated from *O. ochrocephala* and grown on PDA was grey in the middle and surrounded by a brown halo with no conidia (Fig. 5A). *Alternaria gansuense* isolated from diseased stems using a vaccination needle dipped in spore suspension which concentration was 1×106 conidial/ml paddled 4–5 lines in wheat hay decoction agar (WHDA) (Fig. 5B). The great majority of mycelium was grown in the culture medium after culturing for one month, there were a lot of conidia on the colony grown on WHDA, which were long obclavate, distinctly thickened with several transverse septa (Fig. 5C).

The concentration of SW in *A. oxytropis* was significantly higher than those found in *A. gansuense*. SW in *Alternaria oxytropis* was 561.61 μg/g s, the contents of SW were 703.99 μg/g in mycelia of A. oxytropis isolated from locoweed plants *O. ochrocephala*. ($P < 0.05$; Fig. 6).

Peak time of SW in two fungi was between 1 and 2 min, with no towing peak phenomenon (Fig. 7). The target ion obtained in a mass spectrogram of 156.6m/z, was broken with energy of @cid = 25 to receive the SW fragment ion as MH = 174.11 (Fig. 8). The peaks as well as the peak time and target ion of SW in the standard sample were neat and single.

Detection of KS genes in stems and leaves of Astragalus adsurgens

The infection of *A. adsurgens* plants by *A. gansuense* was detected by amplifying a 700bp DNA fragment. The KS gene was detected through electropherogram indicated that all stems and leaves of *A. adsurgens* plants infected by *A. gansuense* contained SW, as well as some of the healthy stems and leaves (Fig. 9).

Detection Of Ks Genes In Plants And Fungus

The KS gene in healthy and diseased *A. adsurgens* plants infected with *A. gansuense* was detected by amplifying a 700bp DNA fragment, indicating that even the healthy-looking *A. adsurgens* plants could contain SW. Both *A. gansuense* and *A. oxytropis* contained SW showing that *A. gansuense* also producing SW (Fig. 10).

Discussion

This study showed that the fungal *Alternaria gansuense* produced swainsonine. The results of this study agree with those of Xu (2007) in which they reported *A. gansuense* systematically infects *A. adsurgens* producing SW (Li 2007; Xu 2017). Although healthy plants also contained SW, the SW content of *A. adsurgens* was correlated with pathogen infection. The correlation between the SW production of *A. gansuense* in vitro and the SW content of *A. adsurgens* plants in the field suggests that *A. gansuense* at least partially controls SW production.

To the best of our knowledge, this is the first report of an SW-producing fungal pathogen caused a systematic disease from a legume. A series of *Alternaria* species are known to occur as endophytes in a variety of plants (Carroll 1988; Petrini and Luginbuhl 1979). As fungal pathogens, *Alternaria alternata* has also been reported as an pathogen infected leaves on Phaseolus (O Donnell J and Dickinson 1980), Alternaria brassicicola infected Arabidopsis in 2003(Wees et al. 2003), but there was no reports of alkaloid-producing.

Cook et al (2017) reported that β-ketoacyl synthase (KS) gene was designed using appropriate domains, which confirmed it was required for SW production(Cook et al. 2017). In this study, KS gene was detected in *A. adsurgens* collected in Heping, Gansu, which agreed with those of Li (2018), which detected a 700bp

KS gene in locoweeds in Tianzhu Tibetan and *A. adsurgens* from Huanxian, Gansu, China (Li 2018). However, the results are different from those of Yu Yongtao, which found less than 0.001% of SW in the infected plants (Yu et al. 2018). The discrepancy may be caused by the high rate of infections of *A. adsurgens* by *A. gansuense* in Lanzhou or may be due to different parts of plant materials used for the study. Our results also showed that KS gene was present in all the *A. adsurgens* plants infected by *A. gansuense* and only in 40% of healthy plants. These results are similar to those of Braun (2003), which showed correlations of the SW content of the locoweed population with *Alternaria* fungus infection (Braun et al. 2003).

The plant pathogen *Embellisia astragali* causes a disease termed YSRR in standing milkvetch (*Astragalus adsurgens*), which was firstly described in 2007(Li 2007). Liu et al (2016) conducted a phylogenetic tree based on the sequences of 3 loci (GPD, RPB2, and TEF-1) strongly supported the placement of *E. astragali* in *Alternaria* sect. Undifilum and *E. astragali* was described as *Alternaria gansuense* comb. nov. because the species epithet “astragali” was already occupied(Liu et al. 2016). We found more content of SW in *A. oxytropis* isolated from *O. ochrocephala* than in *A. gansuense* invaded *A. adsurgens* plants, indicating that SW-producing ability in *Alternaria* pathogen was less than it served as endophyte, in spite of phylogenetic analysis classified the pathogenic fungus *A. gansuense* in the wave bud spore group (Section Undifilium) and has a close relationship with the endophytic fungus (Li Y Z et al. 2011).

O. ochrocephala plants from which *A. oxytropis* were isolated contained significantly more swainsonine than plants from which *A. oxytropis* could not be isolated and the same consequence appeared in trial used KS gene. The results in this study were consistent with those of Cook (2009) which considered *A. oxytropis* found in locoweed plant species, is responsible for the synthesis of swainsonine (Cook et al. 2009a; Cook et al. 2014). *Embellisia oxytropis* was first described in 2006 in locoweed species as responsible for the synthesis of SW (B.M. Pryor et al. 2009), together with *A. gansuense* were *Alternaria* species. Though in this study detected that healthy *A. adsurgens* plants collected in the field contained KS gene, *A. gansuense* was most likely why *A. adsurgens* plants infected by *A. gansuense* containing SW. It is inevitable that looking-healthy plants in the field may infected by *A. gansuense* while no symptoms on the surface, another reason is that *A. gansuense* caused not only a systemic disease but also yellow stunt and root rot disease, symptoms vary in the phenotype of each individual, causing that some of the healthy stems and leaves detected KS gene.

Our results showed that the content of SW in the plant infected with *A. gansuense* was higher than those healthy plants, which could be the reason for the poisoning of animals after feeding on a large number of diseased plants. Research conducted by Stegelmeier showed that doses at or above 0.2 ug/g body weight/day for at least 21 days produced irreversible neurological disease, and lower doses over more extended periods might lead to weight loss and biochemical lesions (Stegelmeier et al. 1999). Previous studies showed that goats fed on *O. ochrocephala* producing about 46.2 μg/g of SW showed poisoning symptoms (Colegate S M et al. 2010; Gu B Q et al. 1990; Wang S et al. 2012). Our analysis shows that the content of SW in *A. adsurgens* ranged from 7.75 to 108.32μg/g, which could easily poison domestic and

experimental animals. This study determined the SW in both healthy stems and leaves in the field using HPLC linked to MS, which confirmed that healthy-looking *A. adsurgens* plants also contained SW probably because the plants were infected with *A. gansuense.* Although the results of this study indicate that content in healthy-looking *A. adsurgens* plants is obviously lower than plants infected with *A. gansuense*, eating a large number of looking-healthy plants in livestock also has a high risk of poisoning.

Declarations

Conflicts of interest

The authors declare no conflicts of interest.

Founding

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Figures

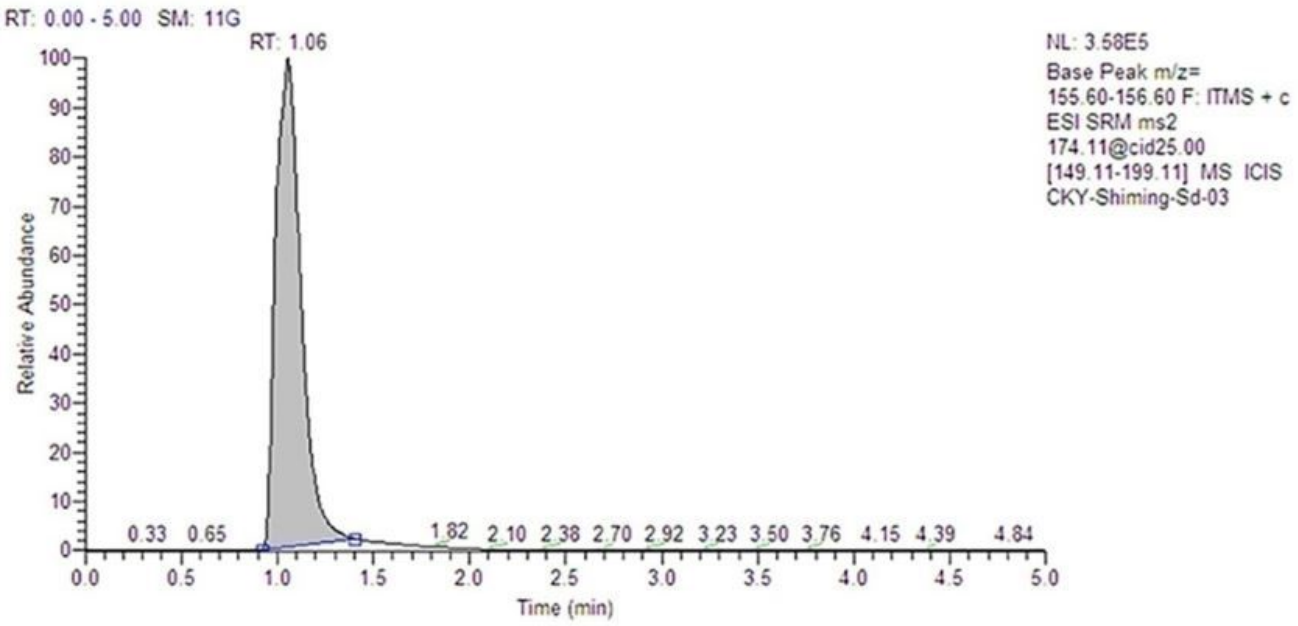

Figure 1

The peak diagram and time obtained with 156.25μg/mL of swainsonine using high-performance liquid chromatography coupled with amass spectrometer and peak emergence time was between 1 and 2 min.

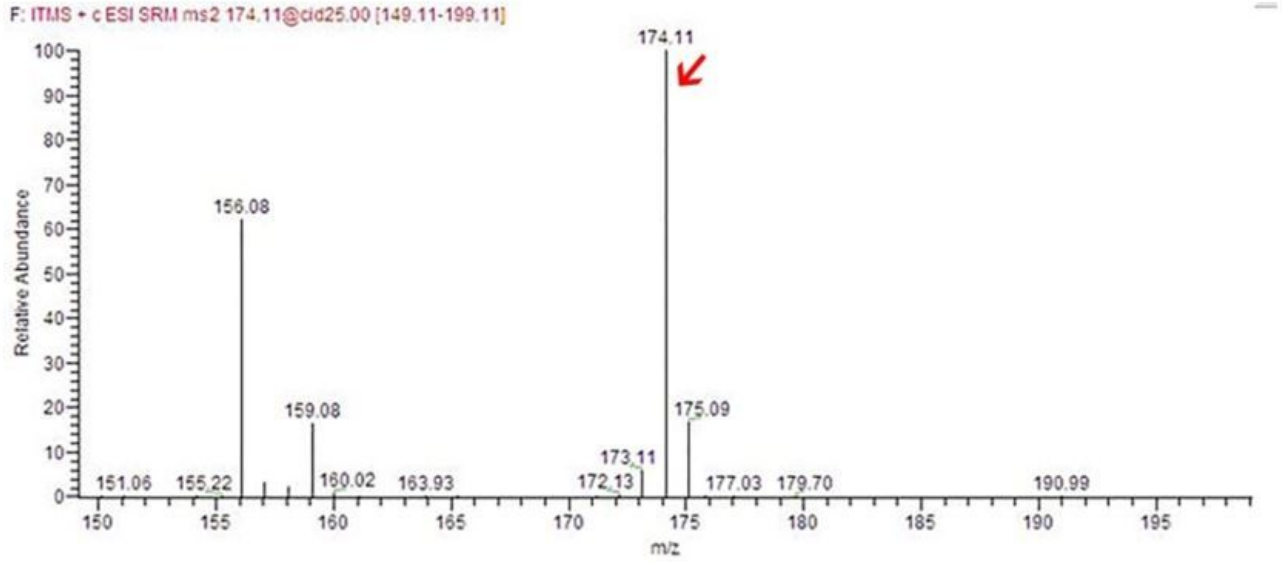

Figure 2

Mass spectrometry of swainsonine in standard samples obtained using high-performance liquid chromatography coupled to mass spectroscopy and the red arrow showed a mass spectrogram of 156.08 by crushing it with the energy of @cid from 25 to a total of 174.11.

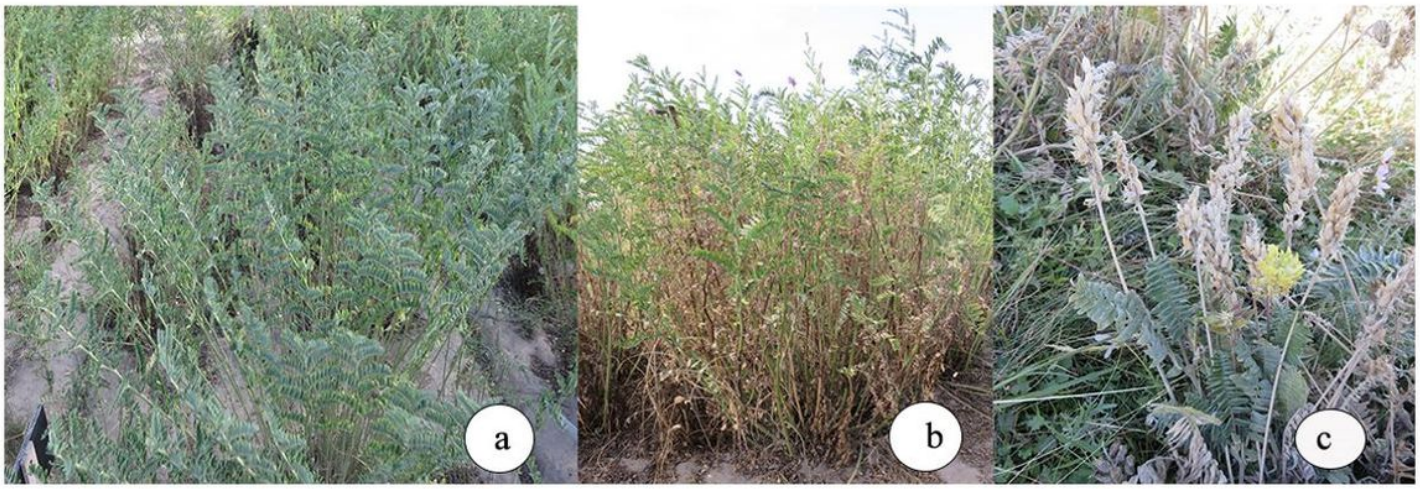

Figure 3

Picture showed field plants. a, healthy *Astragalus adsurgens* plants. b, field plant of *A. adsurgens* infected with *Alternaria gansuense* and c, locoweed of *Oxytropis ochrocephala* plants.

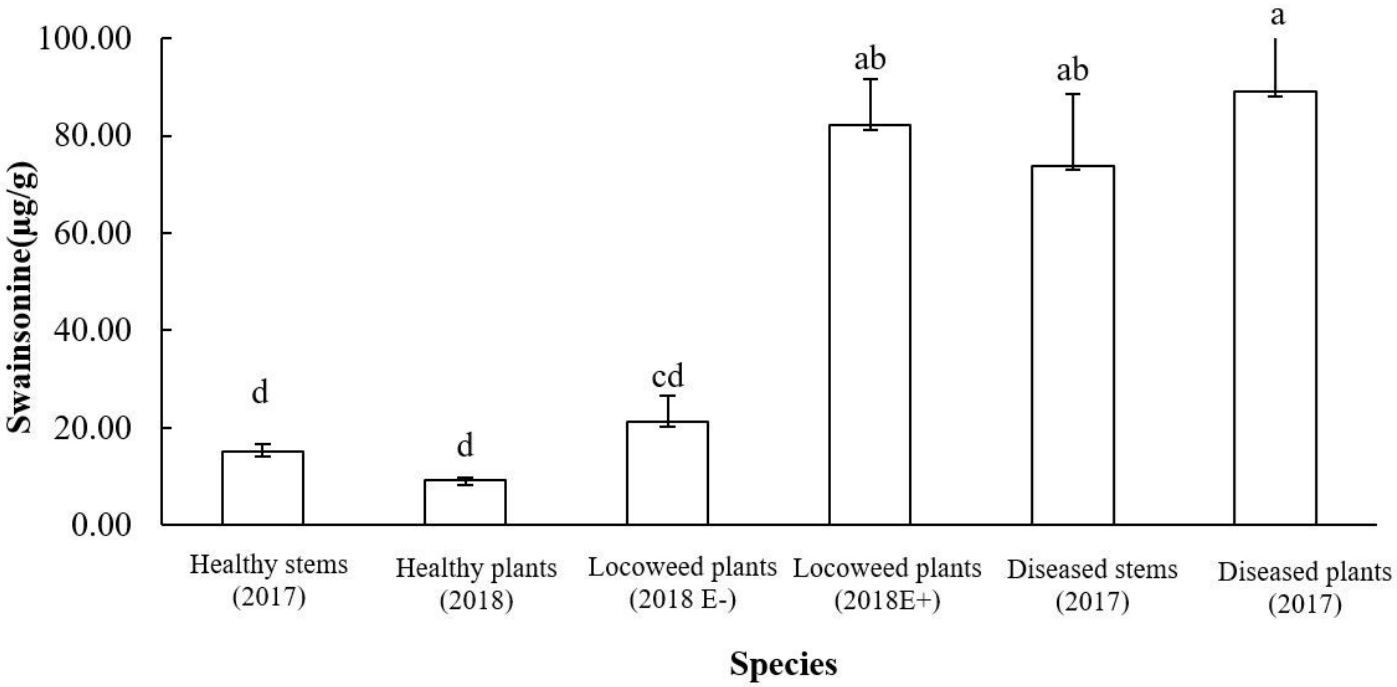

Figure 4

The concentrations of swainsonine (SW) (μg/g) detected in *Astragalus adsurgens*, *Oxytropis ochrocephala* plants collected between 2017 and 2018. Mean SW concentrations were expressed as mean ± standard error. Different letters on the error bars represent significant differences ($P<0.05$).

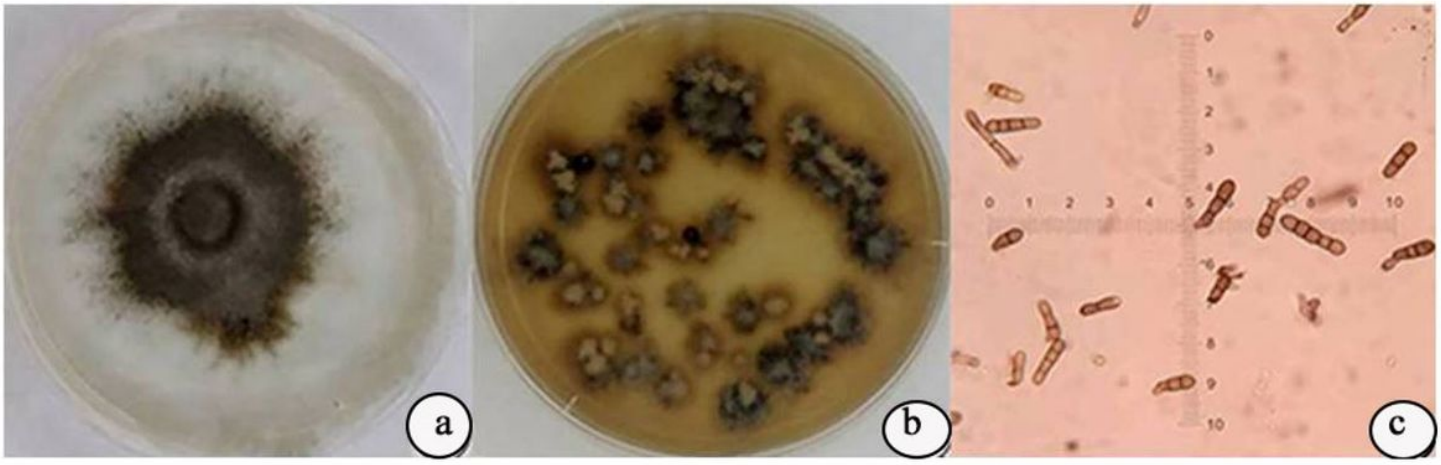

Figure 5

Picture showing pure cultures of fungal colonies isolated from plants. a, *Alternaria oxytropis* isolated from *Oxytropis ochrocephala*. b, *A. gansuense* isolated from *Astragalus adsurgens*. c, Conidia produced upon the colony of *A. gansuense.*

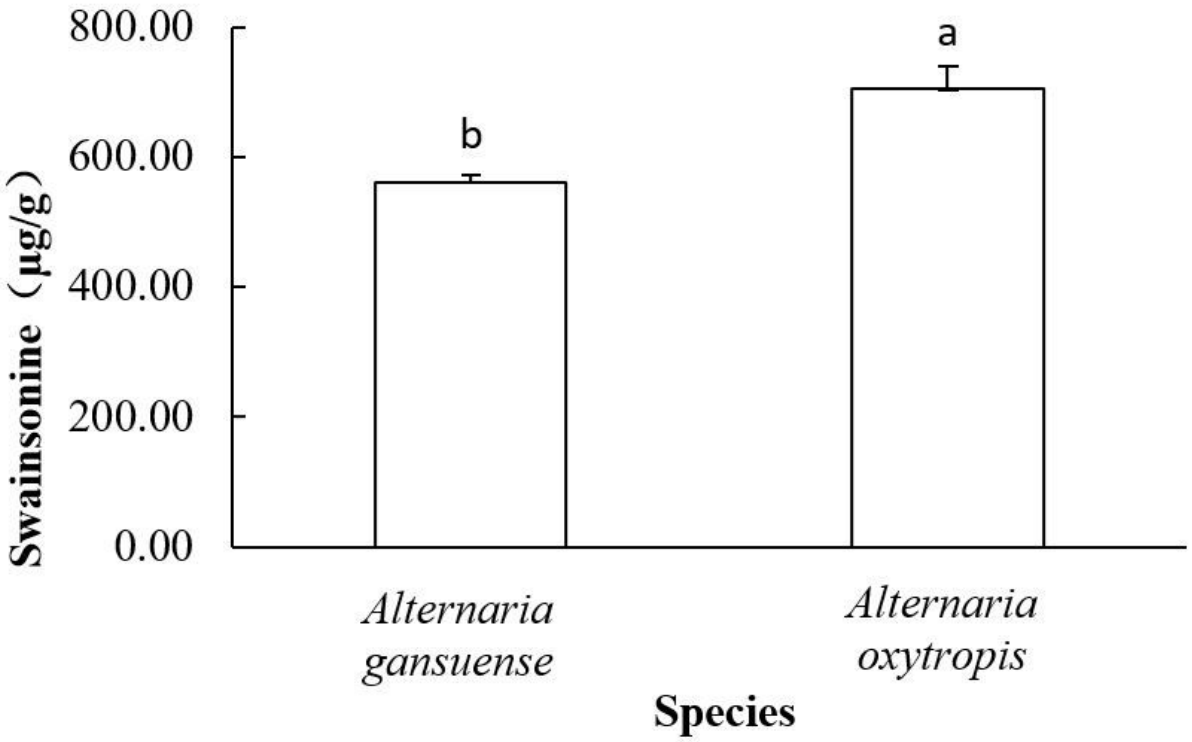

Figure 6

The concentrations (μg/g) of swainsonine detected in *Alternaria gansuense* and *Alternaria oxytropis*. The data were expressed as mean ± standard error. Different letters on the error bars represent significant differences (P< 0.05).

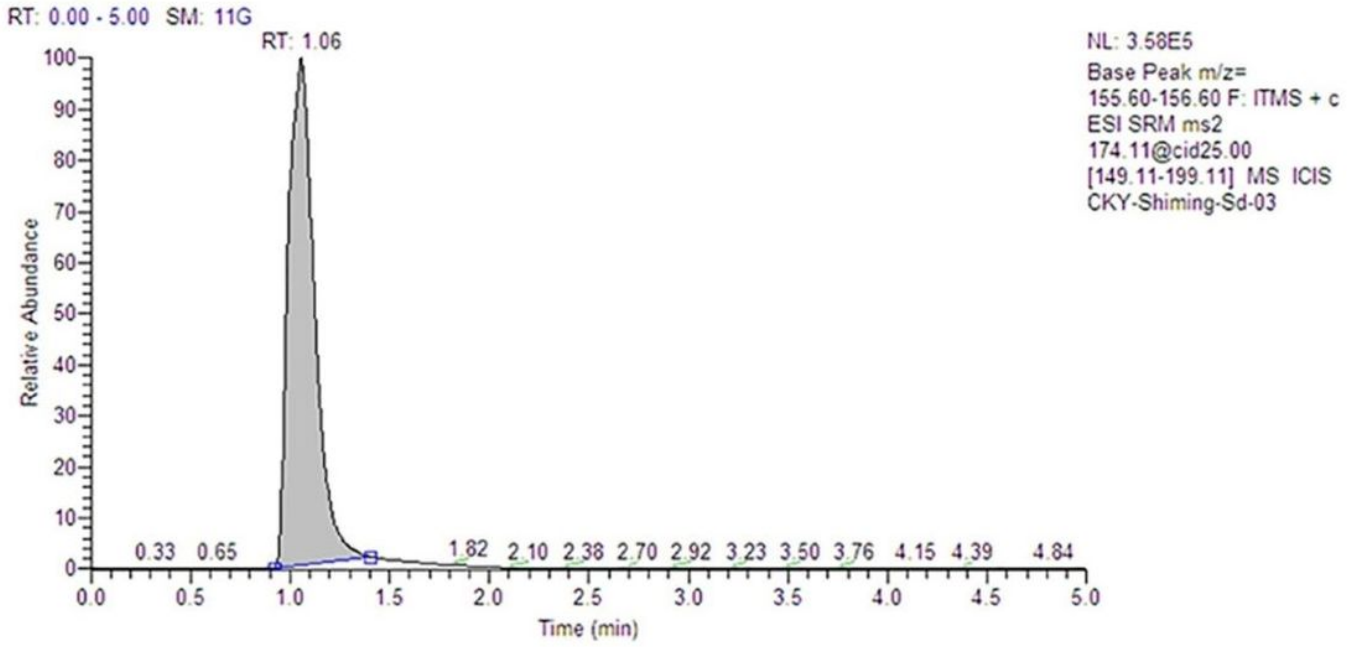

Figure 7

The peak diagram obtained with 156.6 μg/mLof swainsoninein crude extract using high-performance liquid chromatography coupled with a mass spectrometer and peak time of SW in two fungi was between 1 and 2 min, with no towing peak phenomenon.

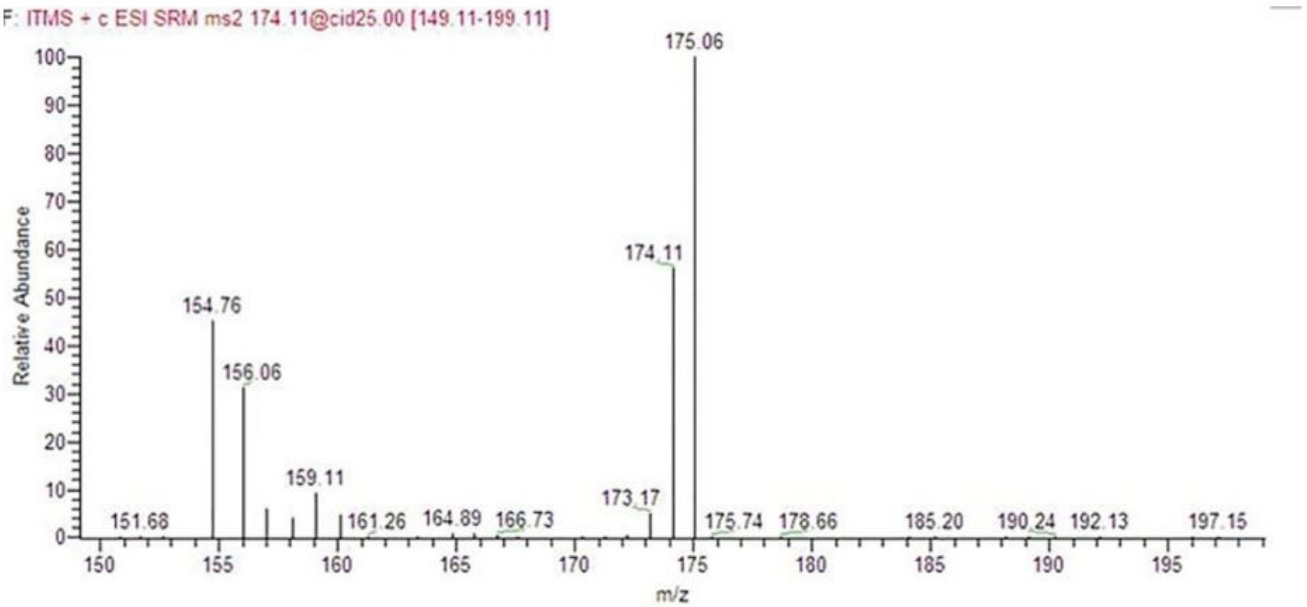

Figure 8

Mass spectrometry of swainsonine in fungi obtained using high-performance liquid chromatography coupled to mass spectroscopy.

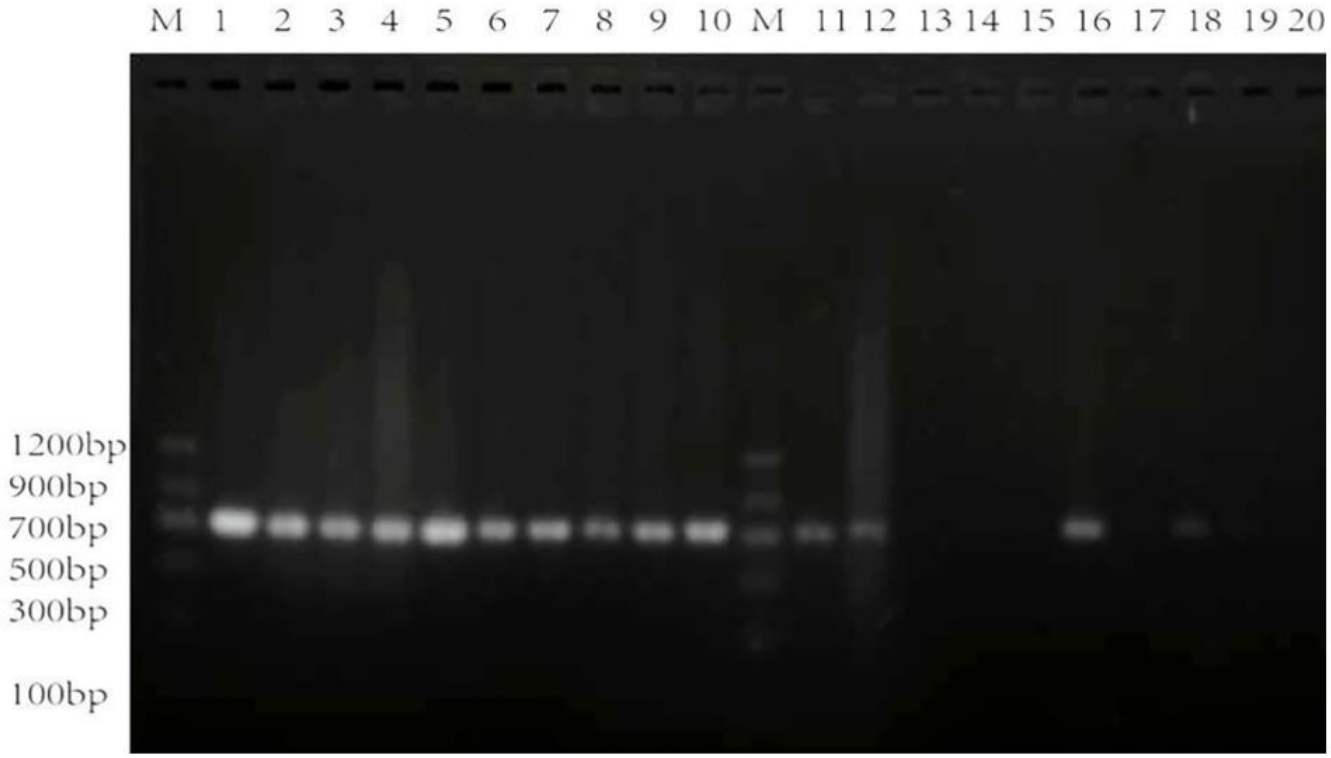

Figure 9

Electropherogram of KS gene amplified with KS primers. M: 1200bp marker (Sangon, Shanghai). Lanes 1, 3, 5, 7, 9 are diseased *Astragalus adsurgens* stems, lanes 2, 4, 6, 8, 10 diseased *A. adsurgens* leaves, lanes 11, 13, 15, 17,19 are the healthy *A. adsurgens* stems, and the lanes 12, 14, 16, 18,20 are the healthy *A. adsurgens* leaves, respectively).

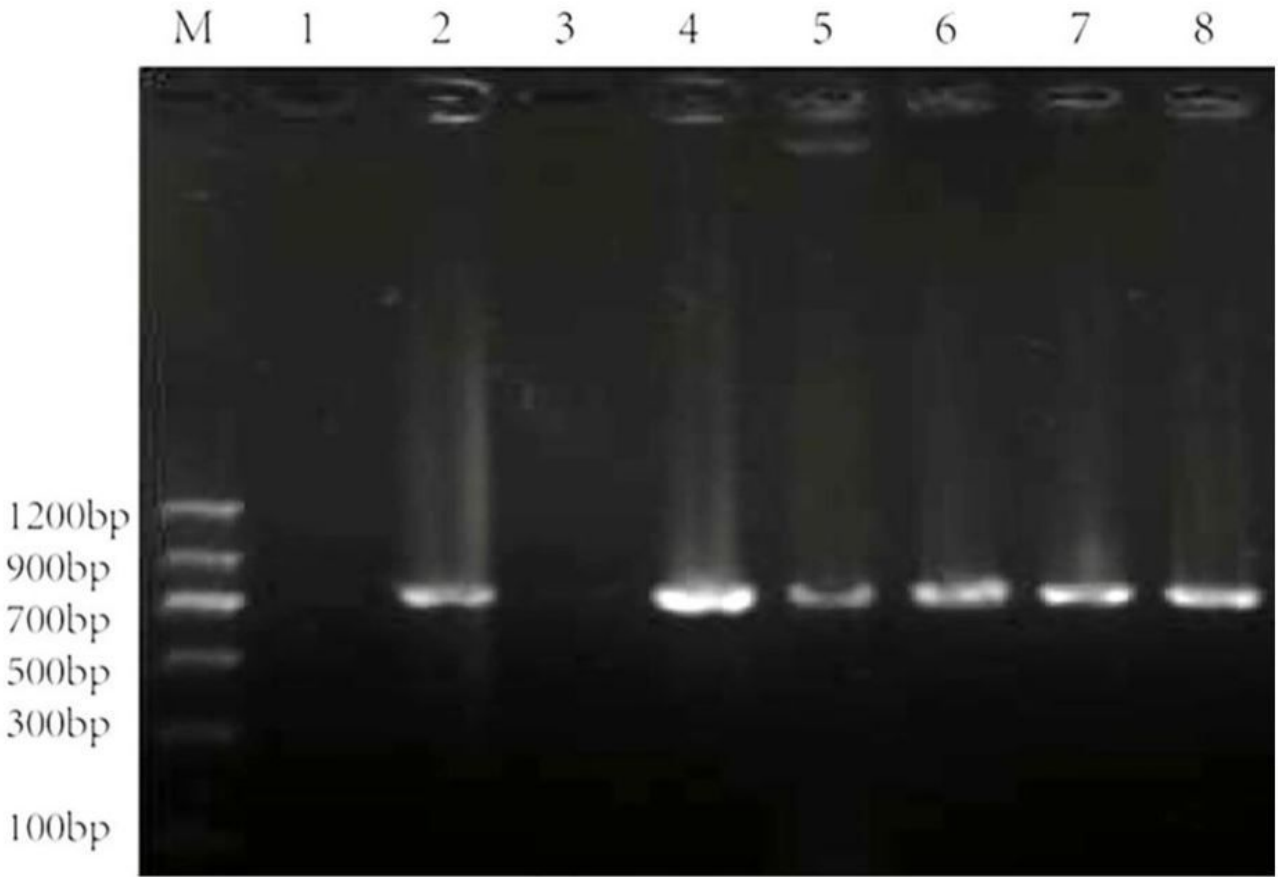

Figure 10

Electrophoresis of PCR amplified KS gene (M: 1200bp marker, swimming lane 1 and 2 are healthy *A. adsurgens* plants; lane 3 is *O. ochrocephala* plants without *A. oxytropis*, lane 4 is *O. ochrocephala* plants with *A .oxytropis*, lane 5 and 6 are diseased *A. adsurgens* plants; lane 7 is *A. gansuense*; lane 8 is *A .oxytropis*).