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Parthenium Weed (*Parthenium hysterophorus* L.) Research in Ethiopia: Investigation of Pathogens as Biocontrol Agents

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Abstract

Parthenium is an exotic invasive weed that now occurs widely in Ethiopia. Surveys to determine the presence and distribution of pathogens associated with parthenium and further evaluation of the pathogens found as potential biocontrol agents were carried out in Ethiopia since 1998. Several fungal isolates of the genus **Helminthosporium, Phoma, Curvularia, Chaetomium, Alternaria, and Eurotium** *were obtained from the seeds and other plant parts. However, all of the isolates tested were non-pathogenic except* **Helminthosporium** *isolates. The two most important diseases were the rust*, **Puccinia abrupta** var. **partheniicola** *and the phyllody, caused by a phytoplasma belonging to the species* ***"Candidatus Phytoplasma aurantifolia"****. Host specificity tests revealed that the rust*, **P. abrupta**, only sporulates *on parthenium while the phyllody infected parthenium, groundnut, sesame, grass pea, lentil, and chickpea. Suspected insect vectors were examined for phytoplasma infection by means of Polymerase Chain Reaction (PCR). The successful acquisition of phytoplasma's by the leafhopper,* **Orosius cellulosus Lindberg (Cicadellidae**), *was determined by molecular detection of phytoplasma. Phytoplasma was also detected from a single bait plant after feeding by the leafhopper. Sequencing data from phytoplasma obtained from parthenium and the above mentioned crops was identical with sequence identities > 98%. The rust was commonly found at 1400 – 2500 m.a.s.l. with disease incidence up to 100% in some locations while phyllody was observed at 900 – 2300 m.a.s.l. with incidence up to 75%. Individual effects of the rust and phyllody diseases on Parthenium in different locations under field condition showed significant reduction on seed and morphological parameters. Seed production was reduced by 42 and 85% due to rust and phyllody, respectively.*

Introduction

Parthenium weed *(Parthenium hysterophorus* L.), here after referred to as parthenium is an herbaceous invasive weed that is believed to be originated in tropical Americas, now occurs widely in India, Australia and East Africa. It is an annual procumbent, diffused leafy herb, 0.5 – 2.5 m tall, bearing alternate, pinnatified leaves, belonging to the family Compositae. It is believed to have been introduced into Ethiopia in 1970s and has become a serious weed both in arable and grazing lands (Taye, 2002).

The major ecological and morphological characteristics that contribute to its severe invasiveness might be its adaptability to wide climatic and soil conditions, production of allellochemicals and the ability to produce large number of seeds (10,000-25,000 per plant) which are small in size (1-2 mm diameter) and light in

weight (50 μg) to travel long distances by wind, water and other means (Navie *et al*., 1996).

Parthenium can cause severe crop yield losses. In India, a yield reduction of 40% in agricultural crops (Khosla and Sobti, 1979) and 90% reduction in forage production in grasslands (Nath, 1988) were reported. In the Caribbean, *Parthenium* is the fourth most serious weed (Hammerton, 1981) while in Kenya it is reported as one of the important weed in coffee plantations (Njoroge, 1991). In eastern Ethiopia, it was reported that *Parthenium* caused sorghum grain yield was reduced from 40 - 97% depending on the year and the location (Tamado, 2001). At present, it is found in 30 zones and more than 120 districts in Ethiopia. Its impact is already significant, and thus, its management is of high concern by the government, researchers and the farmers.

Although there are several methods to control *Parthenium*, each has its own limitations. For instance, removing by slashing or mowing results in regeneration of new shoots that lead to a repeated operation. Manual and mechanical uprooting also prove to be of limited value owing to enormous amount of labour and time required and vulnerability of workers engaged in the operation to the various kinds of allergies caused by the weed. Chemical control, though effective, is temporary and needs repeated application. Hence, the use of biocontrol agents including insects, pathogens and strong interfering smoother crops and plants is recommended for the long-term management of *Parthenium***.**

Surveys for natural enemies were carried out in Mexico by the Common Wealth Institute of Biological Control (now CABI bioscience, Ascot, UK). Eight insect species and two rust fungi were introduced and released in Australia after preliminary screening in Mexico and final evaluation in quarantine in Australia (Evans, 1997). However, the author has indicated that adverse climatic factors have prevented the released natural enemies from achieving their full potential and hence, a longer period of adaptation than originally envisaged may be necessary (Evans 2002). Microorganisms associated with *Parthenium* were also studied in India and such efforts are still in progress by scientists towards the development of indigenous pathogens (Kumar, 1998).

It has been suggested that natural enemies comprised of native pathogens may be more effective than introduced because of more adaptability and no necessity of quarantine measures (Qiumby and Walker, 1982). Besides, surveys for the fauna attacking the weed and determination of their origin in the introduced range is important before introduction of biocontrol agents from abroad. Therefore, the objectives of biocontrol studies carried out were: 1) to collect and identify pathogens associated with *Parthenium* seed, leaf and other plant parts from different locations; 2) to determine the incidence and distribution of diseases; and 3) to determine the effects of potential disease on *Parthenium* morphological parameters and seed production capacity under field conditions; and 4) to determine the host specificity and relatedness of major diseases to economic crops.

Materials and Methods

Isolation and Pathogenicity of Facultative Parasitic Fungi

Samples of parthenium seed and leaf were collected from different locations in Ethiopia (Kobo, Woldiya, Kombolcha, Shewa Robit, Nazareth, Welenchiti, Asebe Teferii, Hirna, Dire Dawa, and Alemaya) in order to isolate fungal pathogens associated with them. The collected samples were stored at 4°C until examination. After disinfecting with 1% NaOCl for 10 minutes, fifty seeds and 50 floral parts of parthenium from each location were laid on Slight Nutrient Agar (SNA) (five seeds or floral part were laid per plate in a circular arrangement). Isolation, characterisation and pathogenicity tests of the selected isolates were performed at 20°C with 14 hours light and 10 hr dark conditions following the methods used by Gossmann and Einhorn (1999). The cultures were examined 7 days after incubation and subjected further to obtain pure cultures on different agar media vis., Slight Nutrient Agar (SNA), Potato Dextrose Agar (PDA) and Oat Meal Agar (OMA) for characterization. Observations were made on colony size, colour, mycelium growth, and formation of conidium, its size, shape and septation. The fungi were identified using the collected data and compared to fungal identification keys and reference books.

Production of spores of putative *Phoma* sp., *Alternaria* sp., and *Trichoderma* sp. isolates were conducted on PDA and OMA while the isolates of *Chaetomium* sp., *Helminthosporium* sp., *Curvularia* sp., *Eurotium* sp. and *Pestalotia* sp. isolates were grown on SNA. Spore suspension from each isolate was established at a concentration of 1 - 6 x 10^6 and then inoculated to Parthenium plants as a spray at different plant growth stages (4 – 6, 8, and 12 leaf stages) and different inoculation conditions (in a conditioner room: temperature 20/25°C, humidity > 90%, 14 hr light and 10 hr dark, and in greenhouse: temperature 10 – 27°C). Plants were maintained in the greenhouse for infection proliferation and checked for symptoms development at an interval of 7 days after inoculation for four weeks.

Host specificity of *Puccinia abrupta* on related crop and weed species

The selection of test plants for inclusion in the host range screening tests was based on the phylogenetic centrifugal testing sequence of Wapshere (1974) and similarities of plant species tested in International Institute of Biological Control (IBBC) by Parker et al. (1994) and Seier et al. (1997). Crop plant species used in host-range studies were three varieties of *Guizotia abyssinica* L. (Kuyu, Esette and Fogera), sesame (*Sesamum indicum* L.), safflower (*Carthamus tinctorius* L.*).* watermelon (*Citrullus vulgaris* Schrad.) and cucumber (*Cucumis sativus* L.). The related weed species were *Xanthium strumarium. Guizotia scabra. Tagetus minuta. Bidens pilosa. Sonchus* sp., *Bidens schimperii* and *Parthenium hysterophorus.*

The plants of each species were selected and then inoculated at 4-6 leaf stages with the uredospores of Ambo isolate based on its relatively high spore germination capacity as assessed during the initial phase of the study. Three-week-old uredospores (Parker et al. 1994) of each isolate were collected from fresh plants in greenhouse and used for inoculation. The spores were gently shaken and mixed with a sterile solution of 0.01% Tween 80 in distilled water. Inoculated plants were

maintained at about 17 °C for about 24 hrs and then placed in the standard glasshouse condition. Host specificity was confirmed both by assessment of macroscopic symptoms with pustule development and also by microscopic examination of inoculated tissues according to the technique of Bruzzese and Hasan (1983) and Parker et al. (1994). Data on the number of leaves with upper and lower surfaces attacked by rust and number of pustules developed on the upper and lower leaf surfaces were recorded and later subjected to analysis of variance using MSTATC software package (SAS Institute, 1986).

Effects parthenium rust and parthenium phyllody on morphological parameters and seed production of parthenium

Assessment of the effects of parthenium and rust was carried out by comparing the diseased and the healthy plant samples collected from two locations, namely, Ambo and Dukem. Two separate plant sample collections were made: 1) for the determination of the effects on morphological parameters and 2) for the determination of the effects on seed production.

Effects on morphological parameters: 50 plants at flowering stage each for rust/phyllody affected and healthy plants were randomly collected diagonally in the crop field from each of the two locations. The morphological parameters of the plant considered were plant height, number of leaves per plant, leaf length, leaf width, number of branches and dry matter weight. Data from different locations were square root transformed and analysed using MSTATC software package. The least significant difference (LSD) test was used to separate the means.

Effects on seed production: Similar to the determination of effects on morphological parameters, 50 plants at maturity stage each for the rust/phyllody infected and healthy plants were randomly collected diagonally in the field at maturity stage at the two locations. Collected plants were placed in polyethylene sack and dried in glasshouse using sun light. The floral parts were threshed and separated from the rest of the plant and weighed. A 5 g sample from the threshed floral part were taken for each treatment and spread onto a sheet of paper and the number of seeds were counted using hand lens.

Occurrence and distribution of parthenium rust and parthenium phyllody

Field surveys were conducted in major parthenium infested areas of Ethiopia: the central farm land and rift valley, South and North Wollo, West and East Hararghe, and East Wollega during 1999-2002 (Figure 1). The incidence of parthenium rust and parthenium phyllody diseases were assessed in cultivated lands, vacant lands and in grasslands. Incidence was assessed as percent of parthenium plants with a disease symptom over the total plants in a 4 m x 4 m plots (16 m^2). Five counts were taken per field and 3-5 fields were assessed at random at interval of 2-3 km per location and then scaled as < 1%, 1-5%, 6-20%, 21-50% and > 50% (PPRC 2000). Data on disease symptoms, habitat, rainfall, temperature and soil data were collected. Diseased plant

samples were also collected, tagged and pressed for later examinations in the laboratory.

Detection of phyllody disease

Polymerase chain Reaction: Diseased parthenium plants with phyllody symptom were collected from different parthenium infested areas of Ethiopia: Mojo, Miesso, Hirna, Kulubi, Dire Dawa, Babile, Feddis, Awash, Woldiya, Kombolcha, Robit and Kobo areas in Ethiopia. The plants were air-dried and then stored at 4 °C. Suspected insect vectors were also collected from different parthenium infested areas. The insects were killed by ethanol and air-dried or preserved in 70% ethanol for later examination.

Extraction of DNA from dried parthenium plant and suspected insect vector was carried out using the phytoplasma enrichment procedure developed by Ahrens and Seemueller (1992). After extraction, the nucleic acid pellet was resuspended in 100 µl of water, and then subjected to electrophoresis in 1% agarose gel using 0.5x TBE as running buffer by adding ethidium bromide (5µl/50 ml) and then visualised by UV transilluminator for the presence of DNA. DNA was amplified by Polymerase Chain Reaction (PCR) using the phytoplasma primer pair P1/P7 and/or fU5/rU3. The primers were derived from highly conserved ribosomal sequences and prime at the 5′ end of the 16S rRNA gene and in the 5′ region of the 23S RNA gene, respectively. They were reported to be universal for phytoplasma detection and amplify a DNA fragment of approximately 1800 bp in length that includes the complete 16S rRNA gene of about 1535 bp in size, the 16S/23S rDNA spacer region of about 250 bp in length, and approximately 50 bp from the 5' end of the 23S rRNA gene (Schneider *et al.*, 1995).

The reactions were performed in 50 µl volume of reaction mixture containing 1µl of the nucleic acid sample, 5 µl of Taq polymerase buffer with $MgCl_2$ (1x), 1 µl of each primer pair, 1 u/µl Taq-DNA polymerase in a total volume of 50 µl water. 35 PCR cycles were conducted in automated Robocycler Temperature Cycler (Robocycler gradient 96). The following parameters were used: preheating at 95 °C for 5 min for the first cycle, denaturation at 95 °C for 30 seconds, annealing at 56 °C for 1 min, and primer extension/polymerisation at 72 °C for 1 min and 30 seconds, and the final polymerisation at 72 °C for 7 min. Control tubes without DNA template were used as negative control while the faba bean phyllody (FBP) and sunnhemp (*Crotalaria juncea*) phyllody (SUNHP) DNA templates were used as positive control. Aliquots of post reaction mixture (10 µl from each sample) were resolved in 1% agarose gel stained with ethidium bromide, and then visualised with UV illumination and the length of obtained DNA fragments was estimated.

Electron microscopy: Stem sections of diseased plants were cut and immersed in 6% phosphate buffered (0.1 M; pH 6.8) glutaraldehyde for overnight at 4°C on a shaker. The tissues were washed three times in phosphate buffer (0.1 M; pH 6.8) each for 30 minutes. Tissues were then post fixed using 1% osmium tetroxide for 3 hrs and then washed. Dehydration was carried out by immersing the tissues in ethanol series. Infiltration was conducted by immersing in propylene oxide following the methods

used in the department of phytomedicine, Humboldt university, Berlin. After infiltration, the tissues were embedded in complete Spurr`s low viscosity medium (soft) at 4 °C overnight, and then transferred into gelatine capsule, and placed in an oven at 70 °C for 12 – 24 h. Ultrathin sections were then cut with a glass knife in an ultramicrotome sections, stained with aqueous 4 % uranyl acetate, counter stained with lead citrate and examined in transmission electron microscope.

Transmission of Parthenium Phyllody Phytoplasma

In order to characterize the potential risk of the parthenium phyllody on Ethiopian crops, transmission studies with leafhoppers and dodder were carried out as an approach for determination of host range of the pathogen and identification of potential vector insects.

Results and Discussion

Isolation and Pathogenicity of Facultative Parasitic Fungi

The fungal flora isolated from seeds, floral parts and leaves from different locations consisted mostly of saprophytic fungi, i.e *Penicillium* sp, *Aspergillus* sp, *Alternaria* sp and *Cladosporium* sp. Putative fungal isolates of *Phoma* sp, *Helminthosporium* sp, *Curvularia* sp, *Chaetomium* sp, *Eurotium* sp *and Fusarium* sp were selected for pathogenicity tests. Most of the selected isolates showed no pathogenic effects while some showed non-specific symptoms on *Parthenium* plants. Only one isolate, *Helminthosporium* sp., that was obtained from fruit production farm at Shewa Robit resulted in a leaf blight symptom similar to the infected samples collected from the field (Figure 1). Nonetheless, repeated inoculation tests of this isolate showed that its virulence was very limited and requires high humidity (> 90%) for infection to occur.

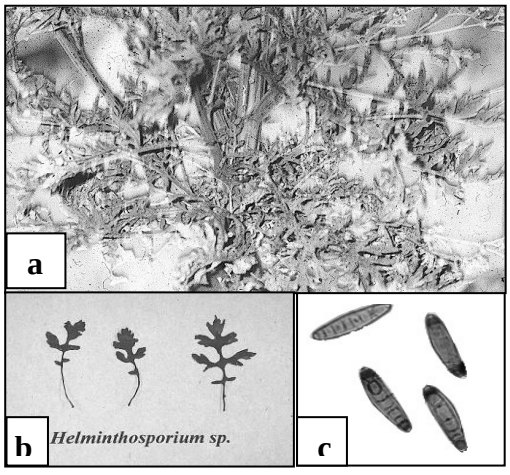


Figure 1. Leaf blight disease caused by *Helminthosporium* sp. collected from Mango field at Shewa Robit: a) symptom of infected plant in the field, b) symptom obtained after pathogenicity test to Parthenium plants two weeks after inoculation; c) spores of *Helminthosporium* sp.

In general, putative pathogenic fungal isolates showed no/non specific symptoms with very limited virulence. It was concluded that these pathogens could be opportunistic with insignificant potential for biological control. It is more than likely that Parthenium weed is merely acting as a secondary host, and, therefore, as an inoculum source for crop diseases (Taye, 2002). In addition, the problem of using such product may be, if it ever reached the production stage, would be the same as for chemical herbicides (Evans, 1997).

Similar to this study, exploitation of local pathogens as bioherbicides for the control of Parthenium has been exhausted in India with limited practical application due to the low degree of virulence; limited distribution, insignificant damage to Parthenium and the tested pathogens have wide host-range and was difficult to consider them as biological control agents (Pandy et al. 1992, Mishra *et al.,* 1994). In Neo Tropics where Parthenium is thought to have originated no efficient non-obligate fungi were obtained (Evans, 1997). Only the downy mildew fungi, *Plasmopara halstedii*, and leaf spot diseases caused by *Alternaria* spp. and *Cercospora partheniphila* were reported to be associated with parthenium with limited distribution and insignificant damage in the Neo tropics (Evans, 1997).

Parthenium Rust

The pathogen that causes rust on *Parthenium* in Ethiopia was identified as *Puccinia abrupta* Diet. & Holw. var. *partheniicola* (Jackson) Parmelee 1967. It was found infecting leaves, stems, and floral parts of *Parthenium* plants in cool and humid areas of Ethiopia. Symptoms revealed on the plant were chlorosis, necrosis, and reduction in vegetative growth, and seed production (Figure 2). Host specificity of *P. abrupta* on related crop and weed species showed that its sporulation was observed only on *Parthenium*. However, limited numbers of poorly developed pustules were observed on varieties of niger seed. No disease symptoms were observed on all other crop and weed species tested. Hence, they were rated as immune or highly resistant.

Subsequent to host specificity test, the effect of rust on morphological parameters and seed production capacity of *Parthenium* at Ambo and Dukem was studied. This showed that dry matter yield at maturity and mean number of seeds per plant were significantly reduced by 24 and 46%, respectively, as compared to healthy plants at Dukem. Similarly, 26% reduction in dry matter yield and 40% reduction in mean number of seeds per plant were obtained at Ambo (Figure 3).

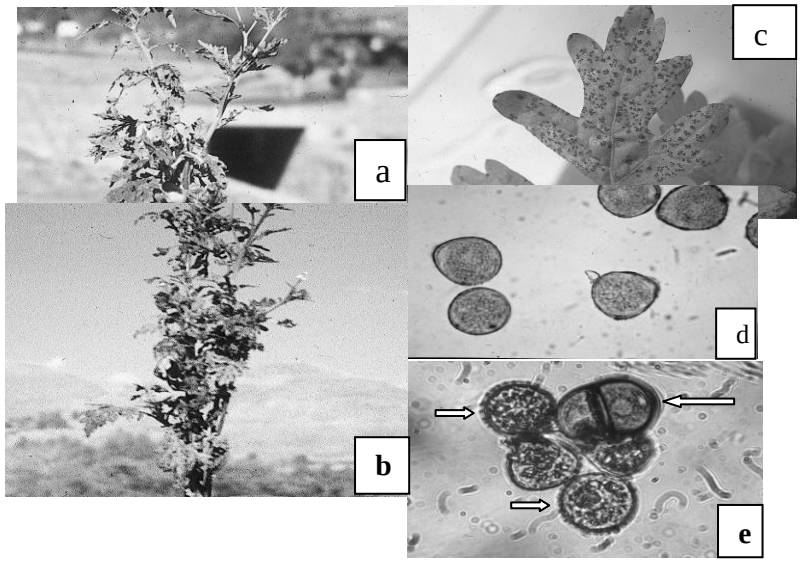


Figure 2. *Puccinia abrupta* var. *partheniicola*: a) and b) infection under field condition; c) pustules with uredospores produced 21 days after inoculation in the greenhouse; d) uredospores; e) teliospore (long arrow) and uredospores (short arrows)

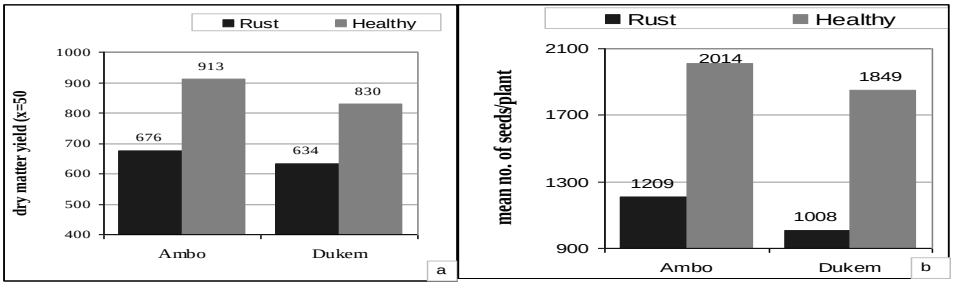


Figure 3. Dry matter yield (g) (a) and number of seed produced per plant (b) by rust-infected and healthy Parthenium plants at Ambo and Dukem

P. abrupta was introduced into Australia in 1994 from Mexico as biological control agent of Parthenium after many screening procedures (Parker *et al*., 1994). However, in Ethiopia, it might be accidentally introduced possibly together with Parthenium from Kenya and/or Somalia (Taye et al., 2004a) for the presence of *P. abrupta* was reported in Kenya in 1977 (Evans, 1987).

It was observed that *P. abrupta* was successfully infecting parthenium in many infested areas with significant reduction on seed production and morphological parameters in the field. The rust was commonly found in cool mid altitude (1500-2500 m) regions of the survey areas where the rainfall varies from 700-1400 mm and the frost occurrence is frequent. This could be due to the requirement of low temperature and leaf wetness in the form of dew for germination of spores and subsequent development of infection. This is in agreement with the result of Parker et al. (1994)

which indicated that low level of pustule formation were produced with dew periods of 4-6 hours while higher levels from 7 hours or more of leaf wetness in greenhouse. In our study, rust incidence is low or does not exist at all in warm temperature areas with the altitude less than 1400 m. This might be due to high night temperatures that result in unavailability of dew that stimulates spore germination. Similarly, Parker et al. (1994) reported that *P. abrupta* occurs only at elevated sites in Mexico (> 1000 m), Kenya (1490 m) and India (930 m) and that it was not recorded from Texas or Madagascar where parthenium occurs at low altitudes.

The reduction in leaf number and leaf area due to *P. abrupta* might reduce the amount of photosynthate produced, which in turn reduces plant height, number of branches that bear flowers and seed. Since parthenium reproduces by seed, rust infection is likely to curtail the seed production to a certain extent and spread of the weed. In addition, rust-infected plants would be subjected to increased stress, and reduced competitiveness to the crop and other native plant species. This result also agrees with the study made by Parker et al. (1994) in the greenhouse through repeated inoculation of the rust. They found that infection with the rust increased leaf senescence, decreased the life span and dry weight, and reduced flower production 10-fold. However, artificial inoculation was failed to reduce plant height, leaf production, or lateral branch formation in their studies.

In the specificity screening tests, all the test crop and weed species in this study were found to be resistant or immune to *P. abrupta*. Only *G. abyssinica* varieties showed limited number of poorly developed pustules. As opposed to this study, Parker et al. (1994) found no development of sorus on *G. abyssinica* but only on *Parthenium conferatum* var. *lyratum* of the 120 species and varieties tested for host specificity. They observed host responses such as necrosis around point of infection, cell wall granulation, callose formation around haustria or on host cell walls around infection or deposition of polyphenols around penetrated stomata in many of the test plants. Additional screening using different climatic and biotic parameters designed to stress or predispose the plants to rust infection also showed no further symptoms appeared and the rust was finally approved by Australian Quarantine and Inspection Service (AQIS) and then released in 1991 (Parker et al. 1994).

In this study, infection of *P. abrupta* on *G. abyssinica* was not observed under field condition. Conducting host range studies in artificial conditions might predispose plants to infection. Hence, the development of symptoms on *G. abyssinica* varieties through artificial inoculation in the greenhouse might arise due to increased disease pressure giving false positive results that would not arise in the field as reported by Evans (2000).

Parthenium Phyllody

Detection of parthenium phyllody disease was conducted through assessment of its symptoms, PCR and RFLP analysis, and electron microscopy of the disease causing agent (Taye 2002, Taye et al, 2004b; Hoppe, 2005; Henninger, 2008; Janke, 2008). Results of each of this method are indicated below:

Symptoms of the Diseased Plants

Phyllody infected plants of *Parthenium* are characterised by their excessive branching, reduced plant height, and transformation of floral parts into many small green leaf-like structures that lead to sterility of plants (Figure 4). Moreover, the diseased plants often formed rosette-like structures through the production of stunted axillary shoots from the crown or nodes of the stem. Phyllody infected plants were observed to be varied in size and form that might be due to different time of infection (Figure 4). Some infected plants were dwarf and bushy whilst others were healthy-looking with abnormal leafy inflorescence that did not set seeds. In some cases the rosettes were evident only at the tips of the stem while in other cases it is conspicuous even from a distance.

PCR and RFLP Analysis

Phyllody disease of parthenium in Ethiopia was first studied in co-operation with the Federal Biological Research Institute (BBA), Dossenheim, Germany. DNA was isolated from all samples and tested using phytoplasma-specific primers P1 and P7. Ten out of 12 samples from different geographic regions showing phyllody symptom resulted in positive and uniform amplification products of about 1800 bp. In another experiment, samples of air-dried and fresh phyllody diseased Parthenium specimens and a suspected insect vector, *Tylorilygus apicalis,* were tested for phytoplasma infection by PCR. Electrophoresis of PCR products showed that amplified DNA fragments obtained from dried specimens correspond to that of fresh plant material. A corresponding band was also amplified from the suspected vector species but not from the healthy plants (Figure 5). The presence of PCR products of the expected size again confirmed the presence of phytoplasma in fresh and dried Parthenium specimens showing symptoms of the disease.

Digestion of the amplified PCR products with *AluI* and *RsaI* restriction endonucleases, respectively, showed uniform restriction profiles of phytoplasma DNA from dried and fresh samples that were different from the phytoplasma DNA obtained from the suspected insect vector. After digestion with *AluI*, Phytoplasma DNA from diseased *Parthenium* plants showed restriction profile that corresponds to Sunnhemp Phyllody Phytoplasma (SUNHP) restriction profile but not to that of Faba bean Phyllody Phytoplasma (FBP) (Figure 6). Digestion with *RsaI*, however, showed similar restriction profiles of phytoplasma DNA of all samples except DNA from suspected insect vector suggesting that *RsaI* was not able to differentiate between FBP and SUNHP Phytoplasma DNA as opposed to *AluI*.

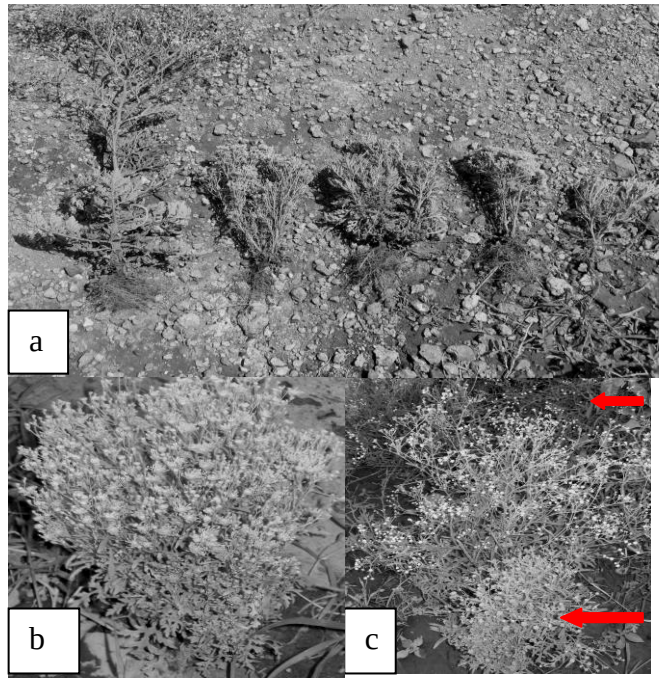
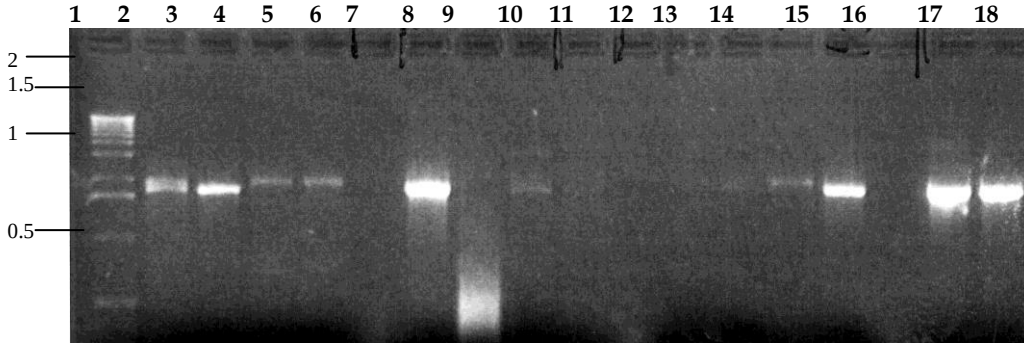


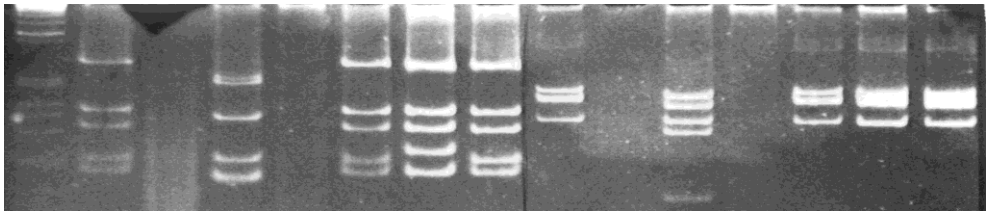
Figure 4. Phyllody disease of parthenium caused by phytoplasma: a) phyllody affected plants showing different level of infection; b) excessive branching and alteration of inflorescence into phylloid structure in which small green leaf-like structures developed; c) diseased parthenium plant (long arrow) and healthy plant (short arrow) with normal inflorescence.



Lane	Sample material	Lane	Sample material
1	DNA marker (1kb, Fermentas)	9	*Tyloryligus apicalis* (Dire Dawa)
2	Phyllody diseased plant (Mojo)	10	Fresh healthy parthenium (Nazareth)
3	Phyllody diseased plant (Hirna)	12	Phyllody diseased plant (Awash)
4	Phyllody diseased plant (Kombolcha)	13	Phyllody diseased plant (Woldiya)
5	Phyllody diseased plant (Kobo)	14	Phyllody diseased plant Kommbolcha
6,11,16	Void	15	Fresh diseased parthenium (Debre Zeit)
7	*Tyloryligus apicalis* (Nazareth)	17	Purified FBP DNA (+ve control)
8	*Tyloryligus apicalis* (Alemaya)	18	Purified SUNHP DNA (+ve control)

Figure 5. PCR amplification of phytoplasma DNA from diseased parthenium and reference samples in faba bean (FBP) and sunnhemp (SUNHP) using the primers P1 and P7

(a)								(b)						
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15



Lane	Sample material	Lane	Sample material
1	DNA marker (1kb, Fermentas)	9	Phyllody diseased plant (Hirna)
2	Phyllody diseased plant (Hirna)	10	*Tyloryligus apicalis* (Nazret)
3	*Tyloryligus apicalis* (Nazret)	11	*Tyloryligus apicalis* (Dire Dawa)
4	*Tyloryligus apicalis* (Dire Dawa)	12	Fresh healthy *Parthenium* (Nazret)
5	Fresh healthy *Parthenium* (Nazret)	13	Fresh diseased *Parthenium* (Debre Zeit)
6	Fresh diseased *Parthenium* (Debre Zeit)	14	Purified FBP DNA
7	Purified FBP DNA	15	Purified SUNHP DNA
8	Purified SUNHP DNA		

Figure 6. *AluI* (a) and *RSA* (b) restriction profiles of ribosomal phytoplasma DNA fragments amplified by PCR assay using universal phytoplasma-specific primer pair P1/P7

A molecular biological analysis of parthenium phytoplasma was further studied by Hoppe (2005), Janke (2008) and Heninger (2008) as part of their MSc thesis. Hoppe (2005) isolated sufficient genomic DNA from infected plants that were free from PCR inhibiting substances. She compared four DNA extraction methods with each other and obtained large quantities of DNA with high molecular weight with PCR inhibitors and small quantities of DNA that ensured the specific PCR reaction. The protocol I from the Nucleo-Spin® Plant Kit produced universal for all plant species DNA preparations, which ensured the PCR reaction (see relatedness of parthenium phyllody to phyllody diseases of economic crops).

A PCR approach which is suitable for molecular detection of phytoplasma in insect and plant samples was established that permits a sensitive detection of phytoplasma within small sample sizes. Because of the incidence of non specific amplifications in every nested PCR sample, sequences of positive nested PCR samples have been analyzed for reliable identification of phytoplasma DNA in insect samples. Due to the high similarity of the sequences from the 16S rDNA-gene, the phytoplasmas detected from suspected insect vectors as well as parthenium are closely related and are part of the same phylogenetic clade "16Sr-IIB" (Candidatus *Phytoplasma aurantifolia*).

Electron Microscopy

Parthenium plants with phyllody symptom were fixed, embedded and ultrathin stem sections of 200 – 300 nm were observed under transmission electron microscope. Phytoplasma-like bodies of different sizes and shapes were detected (Figure 7).

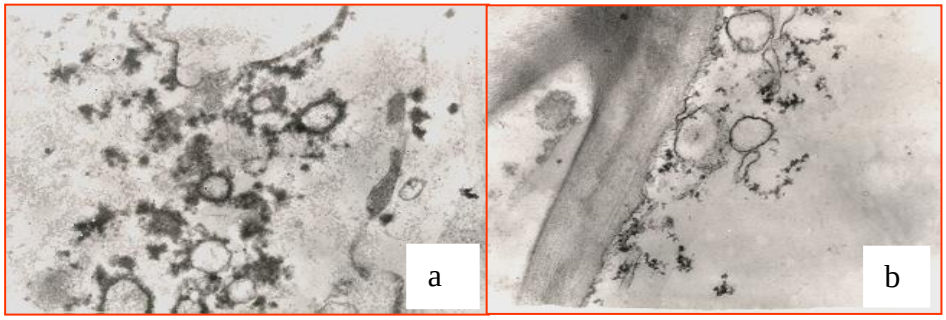


Figure 7. Ultrathin stem sections of Parthenium showing phytoplasmas of different sizes and shapes: a = x 20 000, b = x 25 000

Molecular Detection of Phytoplasma DNA and Transmission by Insects

First attempt of transmission study was carried out indirectly through collection of suspected insect vectors from phyllody infected areas and molecular detection of phytoplasma DNA from insect sample using PCR. Phytoplasma DNA from suspected insect vector *Tylorilygus apicalus.* was detected. However, digestion of the amplified PCR products with *AluI* and *RsaI* restriction endonucleases resulted in restriction profiles of phytoplasma DNA from suspected insect that was different from phytoplasma DNA obtained from diseased Parthenium plants and purified DNA of FBP and SUNHP DNA (Figure 6).

Continued efforts were made by Hoppe (2005) and Henniger (2008) in order to identify the parthenium phyllody vector(s). Hoppe (2005) detected phytoplasma DNA from suspected leafhopper, plant bug, parthenium and selected crop plants. However, similar to Taye (2002), digestion of the amplified PCR products with *AluI* showed that the restriction profiles of phytoplasma DNA obtained from leafhopper and plant bug, were not identical with the phytoplasma DNA obtained from infected parthenium and the selected crops (Figure 8). The results indicate that a phytoplasma associated with the insect is different from *Parthenium* phyllody phytoplasma and the positive control suggesting the phytoplasma obtained from insect may belong to other phytoplasma group.

Henniger (2008) also conducted a detail study on identification of vectors through molecular detection of phtoplasma DNA from suspected insects he collected in parthenium phyllody infested area in Ethiopia as part of his MSc thesis research. The suspected leafhopper and plant bug vectors associated with parthenium were identified at the Institut für Systematische Zoologie, Museum für Naturkunde, Berlin. These were identified at family level based on morphological parameters. They belong to the families of *Cicadellidae, Delphacidae* and *Tettigometridae*. However, identification of the insects to the genus and species level was not completely worked out because of shortage of literatures. The leaf hoppers in the family *Cicadellidae* were identified as *Exitianus* sp., *Cicadulina bipunctella*, *Balclutha* sp., *Agallia* sp., *Orosius* sp., and *Psammotettix* sp. Those in the family *Delphacidae* were identified as *Sogatella* spp. (= *S. vibix* and *S. nigeriensis*) and *Toya* spp (= *Toya propinqua* and *Toya tuberculos groups*). The

plant hoppers were identified to the genus *Hilda* of the family *Tettigometridae* while the plant bug was identified as *Tylorilygus apicalis*.

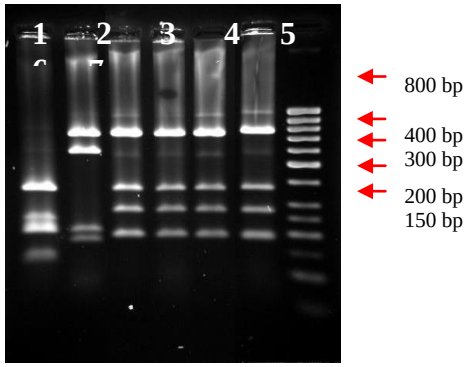


Figure 8. *AluI* restriction profiles of ribosomal phytoplasma DNA fragments amplified by PCR assay using universal phytoplasma-specific primer pair P1/P7. 1 = plant bug, 2 = leafhopper, 3 = Sesame with symptom, 4 = Parthenium with symptom, 5 = peanut with symptom, 6 = *Vinca rosea* (control), 7 = 50 bp Marker, Fa. Fermentas

The leafhopper used in transmission studies was *Orosius cellulosus* Lindberg (C*icadellidae*). A successful acquisition of phytoplasmas by these leafhoppers was proven by means of several positive detections of phytoplasmas in probed leafhoppers. Furthermore, detection of phytoplasmas in a single bait plant suggests that this species is suitable for transmitting phytoplasmas.

Due to the fact that there was no development of phyllody symptoms on bait plants after feeding of phytoplasma infected leafhoppers a successful transmission by *O. cellulosus* has to be reproduced. Besides, members of Tettigometridae were described as potential vectors of phytoplasmas for the first time but a successful transmission of phytoplasma using bioassay test is still has to be proven (Henniger, 2008).

Results from the molecular biological analysis indicate that sesame, peanut, grass pea, chick pea, lentil and chick pea are likely to be infected by the *Parthenium* phyllody causing pathogen. Thus, it could be concluded that Phytoplasmas detected in cultivated crops are closely related, which suggests that parthenium represents a pathogen reservoir for the phytoplasmas affecting agricultural crops in Ethiopia. Since phytoplasma infections can lead to sterility of the inflorescences, severe losses in yield of agricultural crops could be expected.

It is learned that PCR provided a sensitive and rapid means of confirming phytoplasma infection. Such assay can be used to determine the host-range in order to determine the distribution of phyllody phytoplasma on economic crops and related weeds, insect vectors and determine relatedness of phytoplasma diseases in Ethiopia. Therefore, future research works should attempt to determine the phylogenetic positions of the phyllody diseases of economic crops and other weeds in Ethiopia by comparison of their RFLP pattern and sequence analysis of the 16S rDNA with those

of phytoplasma reference strains. The origin of phyllody disease can be assumed to be from collateral hosts (Taye et al., 2004b; Hoppe, 2004; Janke, 2008; Henniger, 2008).

The association of phyllody disease to Parthenium in Ethiopia, India (Mathur and Muniyappa, 1993) and Australia (Navie *et al*. 1996) suggests that Parthenium is susceptible to and naturally affected by phyllody disease in different areas of the world. The susceptibility of Parthenium to phyllody disease suggests that it could be utilized as classical biological control of Parthenium by inducting phyllody using vectors of the pathogen responsible for the disease in order to check its growth and reduce its competitiveness. Releasing the leafhopper vector(s) might control Parthenium plants in vacant lands, roadsides and grasslands. However, the biological control of parthenium with phytoplasma is inadequate due to the following reasons. Firstly, cultivated crops can also be infected. Secondly, the possibility of direct transmission is restricted. And thirdly, phytoplasma-infected parthenium showed no significant reduction of the weed vigour though it reduces seed production by 85%.

Transmission by Dodder

Transmission by dodder, *Cuscuta campestris* was carried out in the greenhouse at Plant Protection Research Centre, Ambo. *Cuscuta campestris* was successfully established on healthy as well as on diseased plants of parthenium. Haustorias predominantly developed on the leaves and leaf-stalks of young plants (Figure 9). But, no characteristic phyllody symptoms on parthenium were induced after twining of dodder from phyllody infected to healthy parthenium and crop plants tested for host range, i.e., lentil, fababean, field pea, haricot bean.

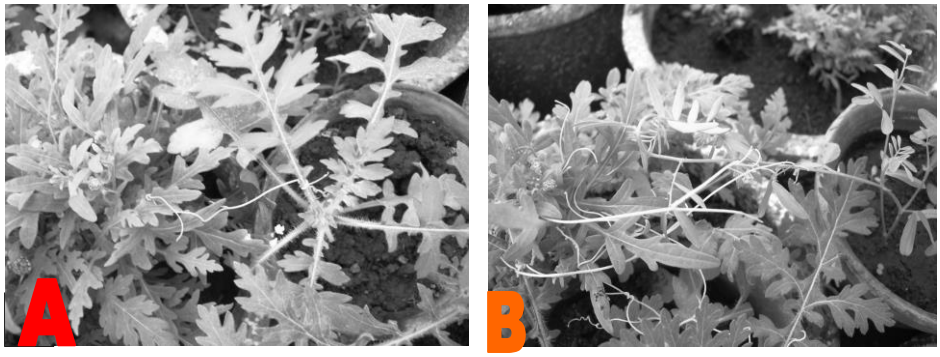


Figure 9. Twining of dodder from phyllody infected parthenium to lentil (a) and to healthy parthenium (b) for transmission of parthenium phyllody phytoplasma

Relatedness of *Parthenium* phyllody to phyllody diseases of economic crops

Symptoms of infected plants: Phyllody infected crop plants were surveyed and collected from parthenium infested areas in the country for molecular detection of phyllody Phytoplasmas using PCR and RFLP analysis of the PCR products. Sesame (*Sesamum indicum*), groundnut (*Arachis hypogaea*), chick pea (*Cicer arietinum*), lentil (*Lens culinaris Medik*), grass pea (*Lathyrus sativum*) and faba bean (*Vicia faba*) were identified with symptoms of phyllody in parthenium phyllody infected areas in Ethiopia.

However, the parthenium phyllody syndrome on faba bean turn out to be due to virus (Figure 10).

PCR and RFLP Analysis

Phytoplasma could be reliably diagnosed with DNA techniques. Parthenium phyllody was detected from parthenium, groundnut, sesame, sesame, chick pea, lentil and grass pea. Phytoplasma rRNA operon parts were extracted and sequenced from groundnut and sesame infected with phyllody disease to verify if the phytoplasma obtained from *Parthenium* was identical with the agent that causes groundnut and sesame phyllody. After digestion with *AluI* enzyme, the amplified PCR products (P1/P7) of parthenium, sesame, peanut, grass pea and *Vinca rosea* infected with faba bean phyllody (FBP) serving as positive control have identical restriction profiles (Figures 11 and 12). These restriction profiles were showing characteristics of witches`-broom diseases.

Sequencing data from phytoplasma obtained from groundnut, sesame, grass pea and *Parthenium* was identical. The data revealed sequence identities, between 98 to 99.8% to phytoplasma within the 16SrII species group (Peanut witches´-broom group) including a phytoplasma originating from papaya, faba bean phyllody (FBP), which serves as type-strain of the Peanut witches`-broom disease, and the reference species *Candidatus* Phytoplasma aurantifolia, causing witches´-broom disease of lime.

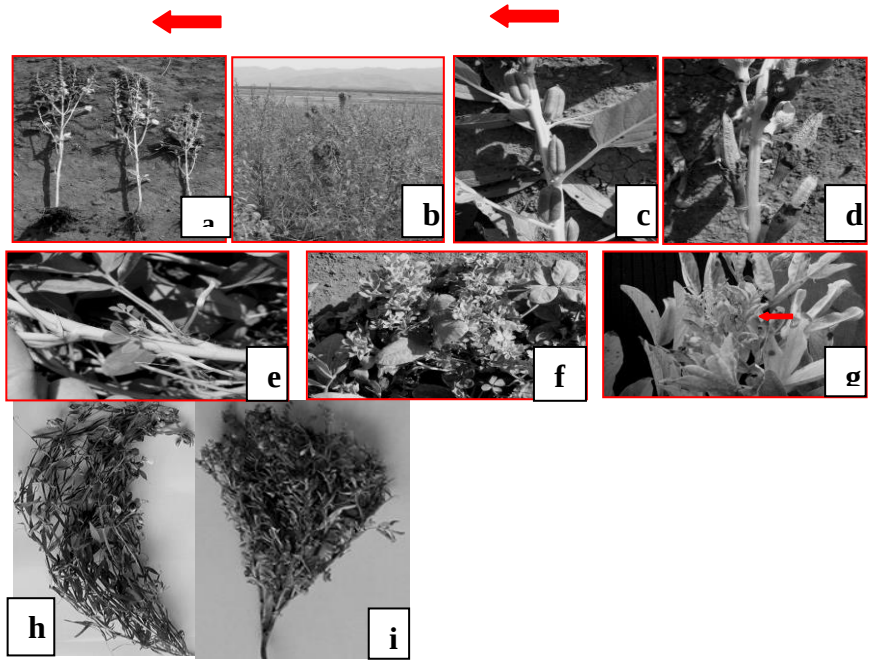


Figure 10. Phyllody infected crop plants: one healthy and two infected sesame plants showing stunted growth and bunchy inflorescence (a); infected sesame plants showing bushy inflorescence in the field (b); pods of healthy sesame plant (c); deformed and shrunk sesame pods containing empty seeds on phyllody infected plant (d); infected groundnut plant with phyllody (e); infected groundnut plant showing small leaves and yellowing (f); infected fababean plant showing sunting, yellowing and small leaves (g); healthy grass pea with fertile flowers (h); and infected grass pea with infertile flowers showing stunted growth and bunchy inflorescence (i)

These crop plants showed extensive phyllody and witches´-broom symptoms, i.e., excessive branching, proliferating shoots, shortened internodes reduced plant height and leaf size, chlorotic leaves as well as modification of floral structures into leaf-like structures that lead to sterility.

Effect of the Phyllody Disease on Morphological Parameters and Seed Production

Mean plant height, leaf length, leaf width and leaf area were significantly reduced while mean number of leaves per plant and number of branches per plant were significantly increased due to phyllody disease. Mean number of leaves per plant of the infected plants was significantly increased by 38 - 69% as compared to healthy plants; mean number of branches per plant by 19 - 35%; and dry matter yield at maturity by 6- 31% over different locations. But, seed production was reduced by 78 - 91% over different locations, indicating the importance of phyllody disease in reducing the reproduction and spread of parthenium (Figure 13).

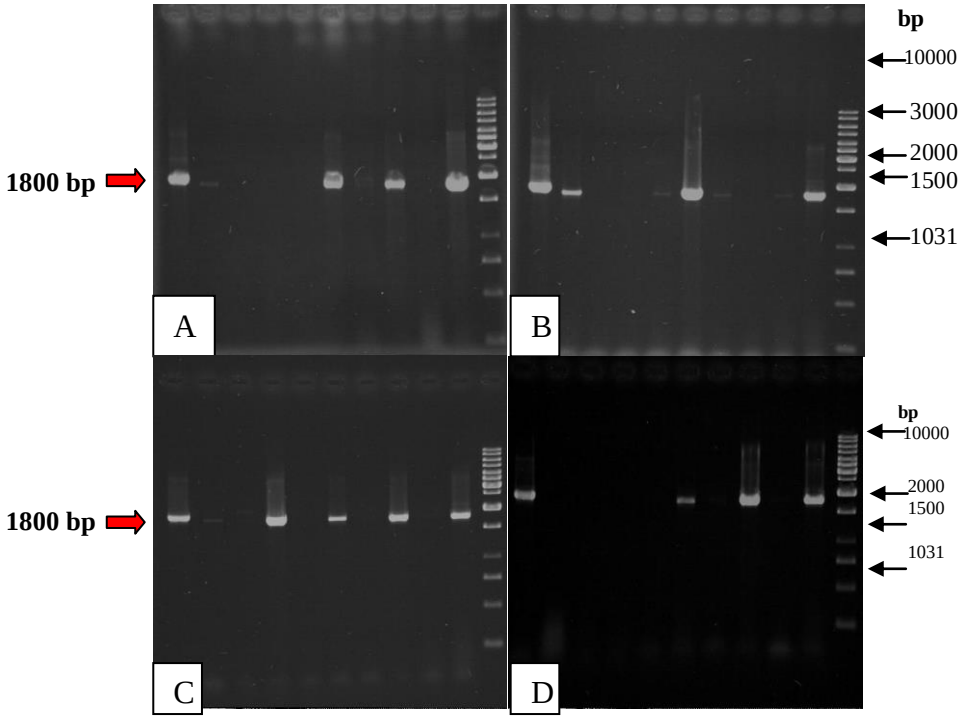
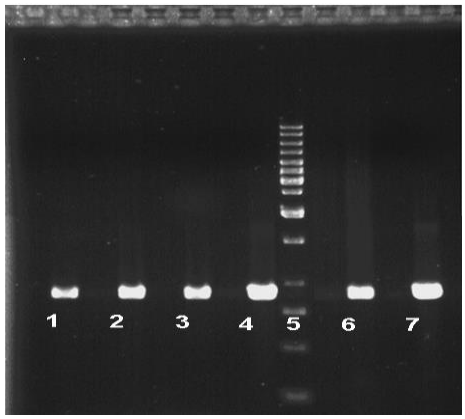
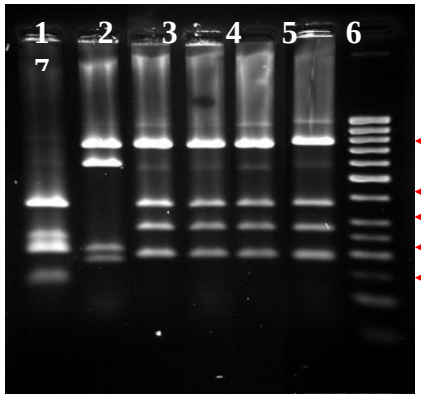


Figure 11. PCR-products amplified using specific phytoplasma primers P1 and P7: DNA-Isolation after Sharma *et al*., 2002 (a), Taye, 2002 (b), MN Protokoll I (c), MN Protokoll II (d) and electrophoresis separation in 1% Agarosegel: 1 = plant bug, 2 = leafhopper, 3 = peanut with out symptom, 4 = peanut with symptom, 5 = sesame with out symptom, 6 = sesame with symptom, 7 = parthenium with out symptom, 8 = Parthenium mit Symptomen, 9 = faba bean with out symptom 10 = faba bean infected with „faba bean phyllody"-Phytoplasma (control), 11 = 1 kb Marker, Fa. Fermentas

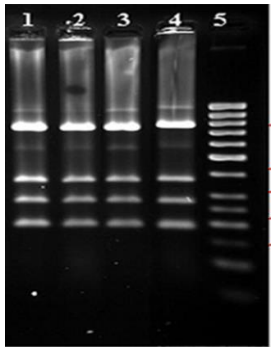


PCR-Amplifications (fU5/rU3; samples 1-4) and nested PCR-Amplifications (P1/P7 and fU5/rU3; samples 6,7) of healthy plants (left) and plants showing phyllody sympomts (right):

1 = Parthenium
2 = Peanut
3 = Sesame
4 = negative and positve control
5 = 1 kb-ladder, Fermentas
6 = Grass pea
7 = negative and positive control



AluI restriction profiles of PCR products after elektrophoresis in 1,5% Agarose gel: 1 = plant bugs, 2 = leafhoppers 3 = Sesam with symptom, 4 = Parthenium with symptom, 5 = peanut with symptom, 6 = *Vinca rosea* (control), 7 = 50 bp Marker, Fa. Fermentas



PCR-RFLP-detection of phyto-plasms; *AluI*-digestion of PCR-Products (P1/P7):

1 = Sesame
2 = Parthenium
3 = Peanut
4 = positive control
5 = 50 bp DNA-ladder, Fermentas

Figure 12. PCR amplifications of phytoplasma DNA extracted from infected crops: *AluI* restriction profiles of PCR products.

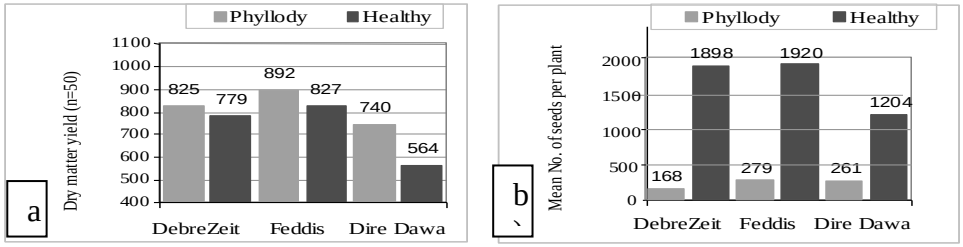


Figure 13. Dry matters yield (a) and number of seed produced per plant (b) by phyllody-infected and healthy Parthenium plants at different locations in Ethiopia

Occurrence and Distribution of Parthenium Rust and Phyllody

The rust was commonly found at 1400 – 2500 m.a.s.l. with disease incidence up to 100% in some locations. The highest incidence (>50%) was observed in the northern parts of the country – in Mersa, Kombolcha, Debre Zeit, Asebe Teferi, Hirna, Kobo, Kersa, and Alemaya. In Weldiya, Addis Mender, Ambo, Hirna, and Kersa the rust incidence was 21–50% both on roadsides and crop fields after harvest. In relatively warm temperature areas like Gobiye, Koka, Nazret, Welenchiti, Harar, and Fedis, incidence was very low (data not shown).

Phyllody was observed at 900 – 2300 m.a.s.l. with incidence up to 75%. The highest incidence of phyllody disease (>50%) was recorded from Robit, Gobiye and Weldiya in North Welo and Anano area in Afar region both during crop growing and fallow period. Incidence of 21–50% was recorded from Kobo, Kombolcha, Addis Mender, Mojo, Nazret, and Kulubi during crop growing period, and in Metehara, Awash, Miesso, and Dire Dawa both during crop growing and fallow period (data not shown).

It seems that phyllody disease is more prevalent in arid and semi-arid low altitude areas than humid cool altitudes in Ethiopia. In some areas like Ambo, Parthenium phyllody was not observed though temperature and rainfall are similar to phyllody prevalent areas. The absence Parthenium phyllody in some survey areas may be due to absence of insect vector(s) that transmits phyllody or else collateral host(s) in the area (Taye, 2002; Taye et al, 2004b).

It is considered that long-term solution will lie on the introduction and releasing of a guild of specific agents, both pathogens and insects, in order to hit as many of the plant organs as possible and thus gradually reduce the weed vigour over time. Up to now there are no potential native insect species observed feeding on Parthenium. Maximum effort should be directed towards importation of host specific insects that are rigorously tested and already in use in Australia through mutual exchange of know-how. These may, however, be extensively tested in quarantine for their feeding on economic plants of Ethiopia and subsequent assessment of the potential benefits and risks, as well as decision making. To name a few, the leaf-feeding beetle, *Zygogramma bicolorata* (Chrysomelidae), a seed-feeding weevil, *Simycronyx lutulentus* (Curculionidae), a stem-galling moth, *Epiblema sternuana* (Torticidae), have been released and successfully established in Australia (Cock and Seier, 1999). Further, the

introduction of microcyclic rust, *P. melampodii*, that occurs in the humid lowland areas of Mexico (Seier *et al*., 1997) to complement *P. abrupta*, which rarely occurs in low land areas of Ethiopia, is recommended after a thorough study of its host specificity. This would greatly reduce the cost of any biocontrol program in Ethiopia, which would otherwise be too expensive (3.5 million Australian Dollar in Australia) to initiate in Ethiopia.

References

Ahrens, U. and E. Seemüller. 1992. Detection of DNA of plant pathogenic mycoplasma like organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA gene. Phytopathology 82: 828- 832.

Bruzzese, E. and E. Hasan. 1983. A whole leaf clearing and staining technique for host-specificity studies of rust fungi. Plant Pathology 32: 335-338.

Cock, M.J., and Seier, M.K. 1999. Biological control of weeds with particular reference to *Parthenium hysterophorus* in Ethiopia. *Arem*: 5: 14-26.

Evans, H.C. 2000. Evaluating plant pathogens for biological control of weeds: an alternative view of pest risk assessment. Australasian Plant Pathology 29: 1-14.

Evans, H. C. 1997. *Parthenium hysterophorus:* A review of its weed status and the possibilities for biological control. *Biocontrol News and Information* 18: 89-98.

Gossmann, M., and G. Einhorn. 1999. Pathogenic fungi on *Chenopodium album* L (fat hen) – Possibilities of biological control. In: Pieber, K. and Bedlan, G. (eds) 2. *Symposium Phytomedizin und Pflanzenschutz im Gartenba*u, Wien, 27-30.09.1999, 55-62.

Hammerton, J.L. 1981. Weed problems and weed control in the commonwealth Caribbean. *Tropical Pest Management* 27: 379-387.

Henniger, T. 2008. Studien zur Übertragbarkeit der Partheniumphyllodie durch Kleeseide sowie Bestimmung möglicher Insektenvektoren. MSc Thesis, Humboldt–Universitat zu Berlin, Landwirtschaftlich-Gartnerischen Fakultat, Fachgebiet Phytomedizin, Berlin, Germany.

Hoppe, B. 2005. Further investigation of biological control of Parthenium (*Parthenium hysterophorus* L.) in Ethiopia. MSc Thesis, Humboldt–Universitat zu Berlin, Landwirtschaftlich-Gartnerischen Fakultat, Berlin.

Janke, J. 2008. Untersuchung zur Phyllodie in Parthenium und zum Wirtskreis der Phytoplasmen in Kulturpflanzen anhand molekularbiologischer Methoden. MSc Thesis, Humboldt–Universitat zu Berlin, Landwirtschaftlich-Gartnerischen Fakultat, Fachgebiet Phytomedizin, Berlin, Germany.

Khosla, S.N. and S.W. Sobti. 1979. Parthenium – a national hazard, its control and utility - review. *Pesticides* 13: 121-127.

Kumar, P.S. (1998). Biological suppression of Parthenium with pathogens. In: Singh, P. S. & Husssani, S.S. (Eds). Biological Suppression of Plant Diseases, Phytoparasitic Nematodes and Weeds. Bangalore, Karnataka, India. pp. 192- 210.

Mathur, S. K. and Muniyappa, 1993. Parthenium phyllody disease in India. In: . Raychaudhuri, S. P. and Teakle, D.S. (eds). Management of plant diseases caused by fastidious prokaryotes: *Proceedings of the fourth Regional Workshop on Plant Mycoplasma*, University of Queensland, Australia, pp. 21 – 34.

Mishra J, A.K. Pandey, R.C. Rajak and S.K. Hasija. 1994. Microbial management of Parthenium: I. Host specificity of *Sclerotium rolfsii* Sacc. National Academy Science Letters, 17: 169-173.

Nath, R. 1988. *Parthenium hysterophorus* L. - A general account. Agric. Rev. 9, 171 – 179.

Navie, S.C., R.E. McFadyen, F.D. Panetta, and S.W. Adkins. 1996. The biology of Australian Weeds 27: *Parthenium hysterophorus* L. *Plant Protection Quarterly* 11: 76 - 87.

Njorge, J.M. 1991. Tolerance of *Bidens pilosa* and *Parthenium hysterophorus* l. To paraquat (gramaxone) in Kenya coffee. *Kenya Coffee*: 56: 999 - 1001.

Parker A, ANG Holden, and A.J. Tomley. 1994. Host specificity test and assessment of the pathogenicity of the rust, *Puccinia abrupta* var. *partheniicola*, as a Biological Control of Parthenium Weed (*Parthenium hysterophorus*). *Plant Pathology* 43: 1-16.

Phatak, H.C., T. Lundsgaard, R. Padma, R. Singh and V.S. Verma, 1975. Mycoplasma-like bodies associated with phyllody of *Parthenium hysterophorus* L. *Phytopath. Z.* 83: 10 – 13.

Pandey, A.K, S. Farkya and R.C. Rajak. 1992. A preliminary evaluation of *Fusarium spp*. for biological control of Parthenium. Journal of Indian Botanical Society, 71: 103-105.

PPRC (Plant Protection Research Centre). 2000. Virus disease incidence assessment methods used in virology section. Plant Protection Research Centre, Ambo, Ethiopia.

Quimby, P.C., and H.L. Walker. 1982. Pathogens as mechanism for weed management. *Weed Science* 30: 30 - 34.

SAS Institute, 1986. SAS/STAT guide for personal computers, version 6 edition.

Seier, M.K., J.L. Harvey, A. Romero, and R.P. Kinnersley. 1997. Safety testing of the rust *P. melampodii* as a potential biocontroll agent of *P. hysterophorus* L. In: Mahadevappa, M and Patil, V.C. (Eds), *Proceedings of the First International Conference on Parthenium Management*, 6 - 8 October 1997, University of Agricultural Sciences, Dahrwad, India, pp. 63-69.

Schneider B, M.T. Cousin, S. Klingkong and E. Seemüller. 1995. Taxonomic relatedness and phylogenetic positions of phytoplasmas associated with diseases of faba bean, sunnhemp, sesame, soybean, and eggplant. Journal of Plant Diseases and Protection 102: 225-232.

Tamado, T. (2001). Biology and management of Parthenium (*Parthenium hysterophorus* L.) in Ethiopia. Doctoral thesis. Swedish University of agricultural Sciences, Uppsala, pp. 1 – 27.

Taye Tessema. 2002. Investigation of Pathogens for Biological Control of Parthenium (*Parthenium hysterophorus* L.) in Ethiopia. PhD Thesis. Humboldt–Universitat zu Berlin, Landwirtschaftlich-Gartnerischen Fakultat, Berlin. 152 pp.

Taye Tessema, G. Einhorn, M. Gossmann, C. Büttner and R.Metz, 2004a. The Potential of Parthenium Rust as Biological Control of Parthenium weed in Ethiopia. *Pest Management Journal of Ethiopia* 8: 83-95.

Taye Tessema, C. Obermeier, G. Einhorn, E. Seemüller and C. Büttner 2004b. Phyllody Disease of Parthenium Weed in Ethiopia. *Pest Management Journal of Ethiopia* 8: 39-50.

Wasphere, A.J. 1974. A strategy for evaluating the safety of organisms for biological weed control. Annals of applied biology 77: 201-211.