Phyllody Disease of Parthenium Weed in Ethiopia

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Abstract

Parthenium hysterophorus L. (Compositae, Helianthae) is an exotic invasive weed currently occurring widely in Ethiopia, India and Australia. Studies on the detection of the parthenium phyllody disease-causing agent, incidence and distribution in the field were carried out during 1999–2002. The disease was commonly observed in low-to-middle altitude areas (900–2350 m.) of Ethiopia with an incidence reaching 75%. The phyllody-infected plants were characterised by excessive branching, reduced plant height, leaf size, and transformation of floral structures into leaf-like structures that lead to sterility. Phyllody-diseased plants were examined for phytoplasma infection by means of Polymerase Chain Reaction (PCR) using universal primers P1 and P7. PCR products of about 1800 bp were obtained after amplification of DNA isolated from fresh and dried parthenium specimens showing phyllody symptoms. Digestion with *AluI* restriction enzyme revealed restriction patterns for all samples corresponding to patterns typically observed for the faba bean phyllody (FBP) phytoplasma group. A phytoplasma was also obtained from a suspected insect vector, *Tylorilygus apicalus*, which was not identical with the phytoplasma DNA obtained from a diseased plant. Analysis of the electron microscope photographs also showed pleomorphic phytoplasma-like bodies of different sizes and shapes. The susceptibility of parthenium to phyllody disease may, therefore, be utilised as a classical biological control of parthenium after assessing the potential benefits and risks.

Key words: Phyllody, Parthenium

Introduction

Parthenium hysterophorus, an invasive weed believed to be originated in tropical America, and currently occurs widely in India, Australia and East Africa. It is an annual procumbent, leafy herb, 0.5–2 m tall, which bears alternate, pinnatified leaves and belong to the family Compositae. In Ethiopia, it has become, since the previous decade, a serious weed both in arable and grazing lands (Tamado 2002, Taye 2002).

Other than competition and allelopathic effect on different crops (Navie et al. 1996) parthenium weed poses health hazard to humans (Kololgi et al. 1997) and animals (Chippendale and Panneta 1994). In India, a yield reduction of 40% on sorghum (Khosla and Sobti 1979) and 90% reduction in forage production in grasslands (Nath 1988) were reported. In eastern Ethiopia, it is reported to be the second most frequent weed (54%) after *Digitaria abyssinica* (63%) and that sorghum grain yield was reduced by 40–97%, depending on the year and the location (Tamado 2002).

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 control operation. Manual and mechanical uprooting was also reported to be of limited value owing to enormous amount of labor and time required and the vulnerability of workers engaged in the operation to the various kinds of allergies caused by the weed (Kololgi et al. 1997). 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 are recommended as components of integrated parthenium management (PAG 2000).

Surveys for natural enemies were carried out in Mexico by the Commonwealth Institute of Biological Control (now CABI Bioscience based on Ascot, UK). Eight insect species and two rust fungi were introduced and released in Australia after a preliminary screening in Mexico and a final evaluation in quarantine in Australia (Evans 1997). However, adverse climatic factors have prevented the released natural enemies from achieving their full potential. Micro-organisms associated with parthenium were also studied in India and such efforts are still in progress towards the development of indigenous pathogens as bioherbicides (Kumar 1998).

Native natural enemies may be more effective than introduced ones because of more adaptability and avoidance of quarantine measures in the case of the former. Since no attempt has been made so far in Ethiopia, this approach can be explored to manage parthenium. Hence, the search for pathogens that cause diseases to parthenium at growth is an essential step for implementating biological control in an integrated parthenium management system in Ethiopia. The objectives of this study were to determine the incidence and distribution of phyllody disease in different infested areas and to detect the phyllody disease-causing agent by PCR and electron microscopy.

Materials and Methods

Occurrence and distribution of parthenium phyllody

Field surveys were conducted in major parthenium infested areas of Ethiopia: the central farmland and Rift Valley, South and North Wollo, West and East Harerge, and East Wellga during 1999–2002 (Figure 1). The incidence of parthenium phyllody diseases was assessed in cultivated lands, vacant lands and in grasslands. Incidence was assessed as per cent of parthenium plants with a disease symptom over the total plants in 4 m x 4 m plots (16 m^2). Five counts were taken per field and 3–5 fields were assessed at random at an interval of 2–3 km per location and then scaled as < 1%, 1–5%, 6–20%, 21–50% and > 50%. Diseased plant samples were 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 during August–November 2001. The plants were air-dried and then stored at 4°C. A suspected insect vector, *Tyloryligus apicalis*, was 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). Accordingly, 0.5–1 g of leaf containing mid-ribs and floral

After extraction, the nucleic acid pellet was re-suspended in 100 µl of distilled and sterile water, and then subjected to electrophoresis in 1% agarose gel using 0.5x as running buffer by adding ethidium bromide (5 µl/50 ml) and then visualised by UV transilluminator for the presence of DNA (Schneider et al. 1995). DNA was amplified by Polymerase Chain Reaction (PCR) using the phytoplasma primer pair P1 and P7, obtained from the Institute of Plant Protection and Fruit Production, Dossenheim, Germany, for all samples. 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 (Ahrens and Seemueller 1992). 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 1 µl of the nucleic acid sample, 5 µl of Taq polymerase buffer with $MgCl_2$ (1x) (stock solution: 10x: 100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, pH 8.3), 4µl of dNTP – mix (1.25 mM each for dATP, dCTP, dGTP, and dTTP), 1 µl of each primer pair (stock solution: 50 µM at the concentration of 50 pmol), 1 u/µl Taq-DNA polymerase (stock solution: 5 U/µl), in a total volume of 50 µl water.

Thirty-five 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 sec, 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 min. Tissues were then post-fixed using 1% osmium tetroxide for 3 hr and then washed. Dehydration was carried out by immersing the tissues in ethanol series. Infiltration was done by immersing in propylene oxide following the methods used in the Department of Phytomedizin, Humboldt University, Berlin. After infiltration, the tissues were embedded in complete Spurr's low viscosity medium (soft) at 4 °C overnight, 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 by a transmission electron microscope.

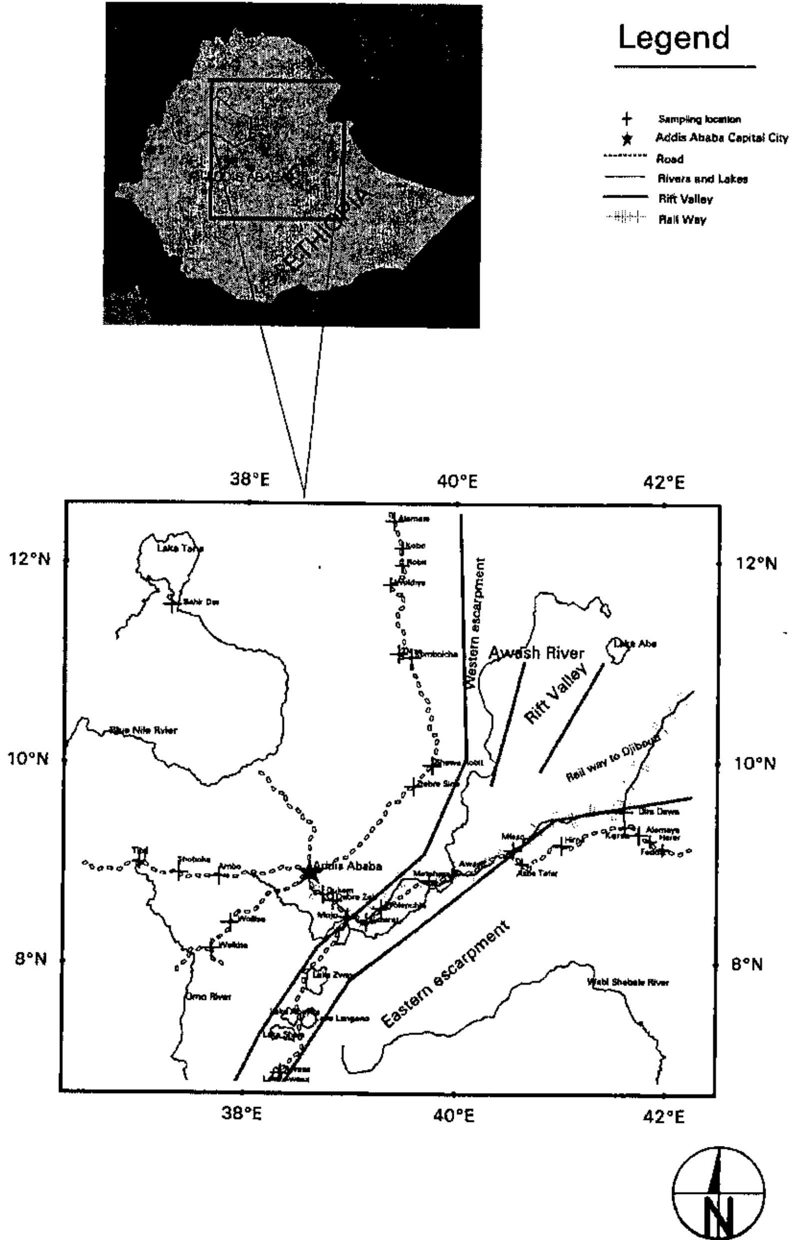

Figure 1. Areas surveyed for parthenium phyllody in northern, central and eastern Ethiopia

Results

Symptoms of the diseased plants

Phyllody-infected plants of parthenium are characterised by their excessive branching,production of stunted axillary shoots from the crown or nodes of the stem. Phyllody-infected plants varied in size and form that might be due to difference in time of infection (Figure 2a). Some infected plants were dwarfed and bushy, while 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. The disease is conspicuous even from a distance in major parthenium-infested areas of the eastern, central and northern parts of Ethiopia.

Incidence and distribution

A survey on the occurrence and distribution of parthenium phyllody disease was conducted in different habitats and in different parthenium-infested locations of Ethiopia (Table 1). Phyllody disease occurred in all altitude ranges of the study areas. It was observed occurring in all surveyed areas except in Sibu Sire, Shoboka, Ambo,Wolliso, Tullubollo, Akaki and Koka. The highest incidence of phyllody disease (> 50%) was recorded from Robit, Gobiye and Woldiya in North Wollo in October 2000, and from Anano area in Afar region both during crop-growing and fallow periods of the same year. 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 periods. In other areas, the incidence range was 6–20%.

Detection of phyllody disease polymerase chain reaction

Twelve parthenium specimens with symptoms suggesting phyllody disease were collected from different locations in Ethiopia in August 2000 and tested for phytoplasma 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 twelve samples from different regions showing phyllody symptom resulted in positive and uniform amplification products of about 1800 bp (Table 2).

In another experiment, samples of air-dried and fresh phyllody-diseased parthenium specimens and a suspected insect vector, *apicalis,* were tested for phytoplasma infection through PCR. Electrophoresis of PCR products showed that amplified DNA fragments were obtained from dried specimens that corresponded to that of fresh plant material (Figure 3). A corresponding band was also amplified from the suspected vector species, but not from the healthy plants (Lanes 7 and 9, Figure 3). Lane 9 showed low concentration of amplified product while Lane 8 showed unclear result (Figure 3). 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.

Restricted fragment length polymorphism (RFLP) analysis

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 (Figure 4). After digestion with *AluI*, Phytoplasma DNA from diseased parthenium plants (Lanes 2 and 6) showed restriction profile that corresponded to sunnhemp phyllody phytoplasma (SUNHP) restriction profile, but not to that of

fababean phyllody phytoplasma (FBP). Digestion with *RsaI*, however, showed not similar restriction profiles of phytoplasma DNA of all samples except DNA from the suspected insect vector; i.e., *RsaI* was able to differentiate between FBP and SUNHP Phytoplasma DNA as opposed to *AluI*. Phytoplasma DNA from insect sample had different restriction profiles from those of diseased parthenium plants and purified DNA of FBP and SUNHP DNA when digested both with *AluI* and *RsaI*.

Electromicroscopy

Parthenium plants with phyllody symptom were fixed, embedded and ultrathin stem sections of 200 from all samples 300 nm were observed with using. Phytoplasma-like bodies of different sizes and shapes were detected (Figure 5). Analysis of the electron microscope photographs in the National Plant Breeding and Phytopathology Institute at Aschersleben, Germany (Dr. F. Ehrig, pers. commu.) also showed the pleomorphic, phytoplasma-like agent.

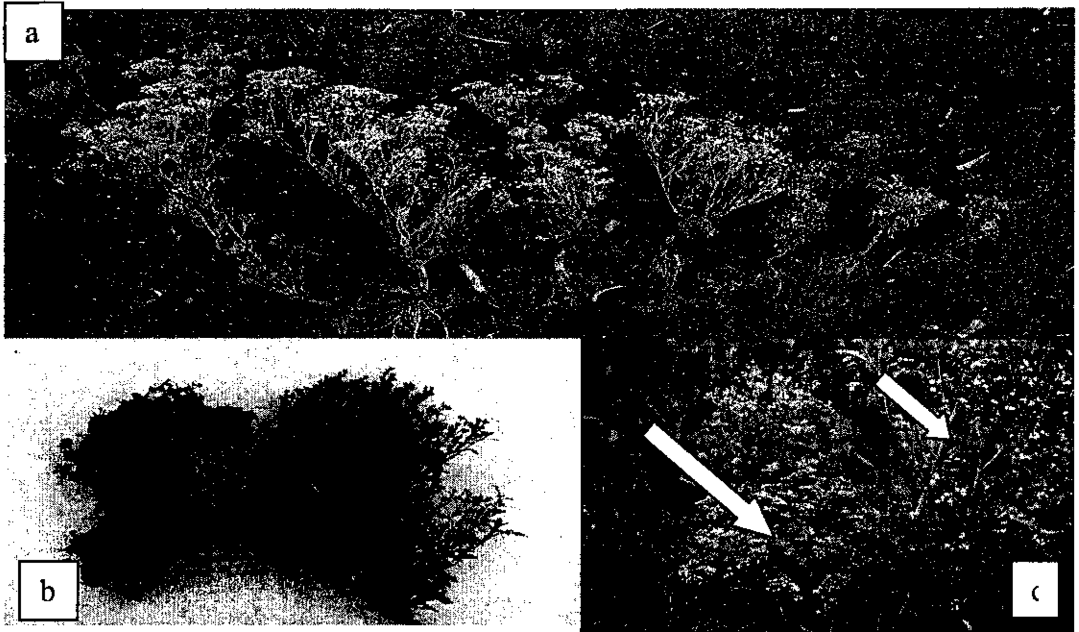

Figure 2. Phyllody disease of parthenium caused by phytoplasma: a) phyllody affected plants showing different severity levels; b) excessive branching and alteration of inflorescence into phylloid structure in which small green, leaf-like structures developed; and c) diseased parthenium plant (long arrow) and healthy plant (short arrow) with normal inflorescence.

Table 1. Incidence and distribution of phyllody diseases of parthenium in northern, central and eastern parts of Ethiopia during January–February and October–November 2000.

Zone	Location	Altitude (m)	Habitat	Phyllody incidence (%)	
				Jan– Feb 2000	Oct – Nov 2000
North Welo	Kobo	1470	Sorghum	6 – 20	21 – 50
	Robit	1500	Sorghum, and maize	21 – 50	> 50
	Gobie	1580	Sorghum	6 – 20	> 50
	Woldiya	1880	Sorghum, grassland	6 – 20	> 50
	Mersa	2300	Roadside	1 – 5	1 – 5
South Welo	Kombolcha	1903	Maize	6 – 20	21 – 50
	Addis Mender	1480	Maize	-+	21 – 50
East Welega	Sibusire	1750	Roadside	-	-
West Shewa	Shoboka	1750	Roadside	-	-
	Ambo	2225	Maize	-	-
	Wolliso	2000	Roadside	-	-
	Tullubollo	2100	Roadside	-	-
East Shewa	Akaki/Dukem	2050	Roadside	-	-
	Debre Zeit	1900	Roadside	-	6 – 20
	Mojo	1870	Maize, roadside	-	21 – 50
	Qoqa	1600	Roadside	*	-
	Nazret	1620	Maize, roadside	-	21 – 50
	Wolanchitti	1450	Maize, tef	6 – 20	6 – 20
	Matahara	970	Maize, sorghum	21 – 50	21 – 50
Afar	Awash	920	Sorghum	21 – 50	21 – 50
	Anano	1200	Sorghum	> 50	> 50
	Miesso	1400	Sorghum	21 – 50	21 – 50
West Harerge	Asebe Teferi	1900	Maize, sorghum	6 – 20	6 – 20
	Hirna	2050	Roadside, grassland	-	6 - 20
	Chelenko	2350	Roadside	6 – 20	6 – 20
	Qobo	2250	Roadside	6 – 20	6 – 20
East Hararghe	Qulubii	2000	Maize, sweet potato	-	21 – 50
	Qarsa	2000	Sorghum, roadside	6 – 20	6 – 20
	Dire Dawa	1360	Sorghum	21 – 50	21 – 50
	Tony farm	1260	Fruit	6 – 20	6 – 20
	Alemaya	2125	Maize and sorghum	6 – 20	6 – 20
	Harar	2100	Roadside	6 – 20	6 – 20
	Feddis	1700	Vegetable	6 – 20	6 – 20

+ - = not observed: * = not assessed

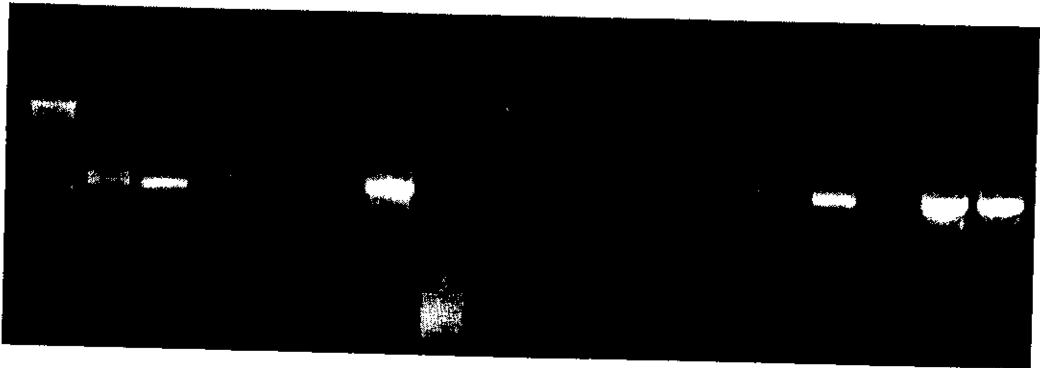

Figure 3. PCR amplification of phytoplasma DNA from diseased parthenium and reference samples in faba bean phyllody phy to plasma (FBP) and sunnhemp (SUNHP) phyllody phy to plasma using primers P1 and P7.

Lane	Sample material
1	DNA marker (1kb, Fermentas)
2	Phyllody-diseased plant (Mojo)
3	Phyllody-diseased plant (Hirna)
4	Phyllody-diseased plant (Kombolcha)
5	Phyllody-diseased plant (Kobo)
6,11,16	Void
7	*Tyloryligus apicalis* (Nazareth)
8	*Tyloryligus apicalis* (Alemaya)

Lane	Sample material
9	*Tyloryligus apicalis* (Dire Dawa)
10	Fresh healthy parthenium (Nazeret)
12	Phyllody-diseased plant (Awash)
13	Phyllody-diseased plant (Woldiya)
14	Phyllody-diseased plant Kommbolcha
15	Fresh-diseased parthenium (Debre Zeit)
17	Purified FBP DNA (+ve control)
18	Purified SUNHP DNA (+ve control)

Discussion

In this study, the incidence of phyllody disease varied between 6 and 75% across different locations, both during fallow and cropping seasons. Similarly, Mathur and Muniyappa (1993) reported that incidence of the disease near Bangalore, India, varied from less than 20% (March–May) to over 70% (October–December) in 1988. In Ethiopia, it seems that phyllody disease is more prevalent in arid and semi-arid low altitude areas than humid cool altitudes. The disease was found having equal incidence in different habitats of the same location, indicating its uniform spread in different habitats such as on roadsides, grassland and crop field. In some areas like Ambo, parthenium phyllody was not observed though temperature and rainfall were similar to those phyllody prevalent areas. The absence of parthenium phyllody in these areas may be due to the absence of insect vector(s) that transmit(s) phyllody agent and/or the absence of alternative host(s) in the area.

PCR and RFLP analysis of the PCR amplified products proved suitable for phytoplasma differentiation and preliminary classification. Other studies reported that the use of additional enzymes allows a more detailed differentiation. Seemueller et al. (1994) indicated that RFLP-based phytoplasma classification using only a few enzymes for analysis does not always coincide with classification based on full-length sequences of the conserved 16S-rDNA gene. The sequence of a large molecule such as the

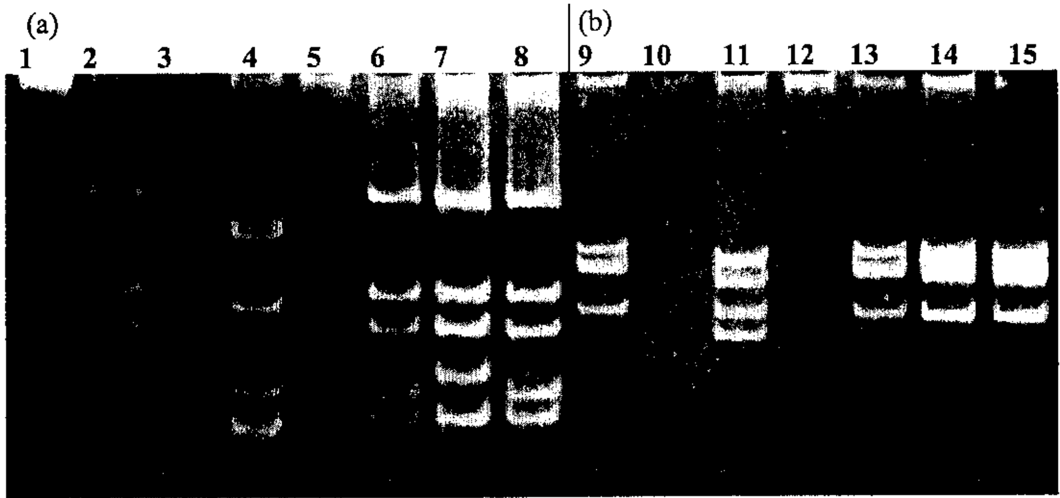

Figure 4. *AluI* (a) and *RSA* (b) restriction profiles of ribosomal phytoplasma DNA fragments amplified by PCR assay using universal phytoplasma-specific primer pair P1/P7.

Lane	Sample material
1	DNA marker (1kb, Fermentas)
2	Phyllody diseased plant (Hirna)
3	*Tyloryligus apicalis* (Nazareth)
4	*Tyloryligus apicalis* (Dire Dawa)
5	Fresh healthy parthenium (Nazareth)
6	Fresh diseased parthenium (Debre Zeit)
7	Purified FBP DNA
8	Purified SUNHP DNA

Lane	Sample material
9	Phyllody diseased plant (Hirna)
10	*Tyloryligus apicalis* (Nazareth)
11	*Tyloryligus apicalis* (Dire Dawa)
12	Fresh healthy parthenium (Nazareth)
13	Fresh diseased parthenium (Debre Zeit)
14	Purified FBP DNA
15	Purified SUNHP DNA

16S rRNA gene reflects phylogenetic distance more accurately than restriction patterns, which depend on significantly fewer genetic characters. Thus, it can be suggested that characterisation and classification of parthenium phyllody phytoplasma examined in this study may be improved by RLFP analysis using additional enzymes and by sequence analysis of the 16S rDNA.

The results of the present study supports the findings of an electron microscopic study by Phatak et al. (1975) and transmission experiments by Mathur and Muniyappa (1993) who observed similar symptoms on parthenium plants following leafhopper vector, *Orocius albicinctus*, inoculation with phytoplasma agent.

However, it is possible that parthenium phyllody agent studied in India could be a different strain of phytoplasma from that detected in the current study even if the diseased plants showed similar symptoms. Hence, comparison of restriction profiles and sequence analysis of parthenium phyllody phytoplasma in India and Ethiopia might facilitate future exchange and utilisation of parthenium phyllody as biological control of parthenium weed.

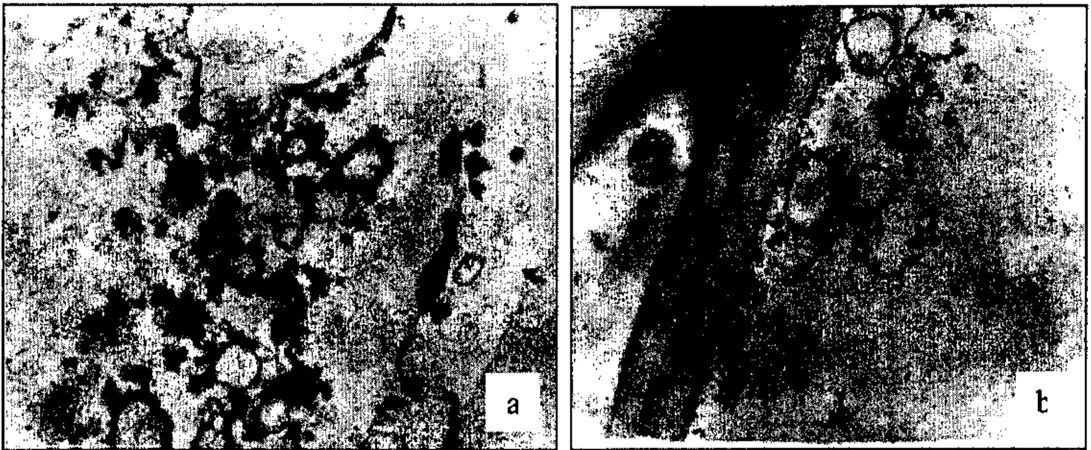

Figure 5. Ultrathin stem sections of parthenium showing phytoplasmas of different sizes and shapes: a = x 20 000, b = x 25 000

Table 2. Result of PCR using universal primer pair P1/P7

Sample no.	Location	Altitude (m)	Result*
D-242.1	Mojo	1870	+
D-242.2	Miesso	1400	+
D-242.3	Hirna	2050	+
D-242.4	Kulubi	2000	+
D-242.5	Dire Dawa	1260	+
D-242.6	Awash National Park	926	-
D-242.7	Woldiya	2010	+
D-242.8	Kombolcha	1903	+
D-242.9	Robit	1500	-
D-242.10	Kobo (Wollo)	1470	+
D-242.11	Qarsa	2000	+
D-242.12	Alemaya	2125	+

* The + sign represents positive result and – negative result

Phytoplasma DNA was detected from the suspected insect vector, *T. apicalis,* in Ethiopia. However, the primer set used in the study was not specific to amplify DNA of FBP phytoplasma group. Digestion with *AluI* and *RSAI* showed that the phytoplasma DNA restriction profiles were not similar to those obtained from infected parthenium plants or purified FBP and SUNHP phyllody phytoplasma strains. This indicates that a phytoplasma associated with the insect is different from parthenium phyllody phytoplasma

and the two positive controls. Thus, the phytoplasma detected from the putative vector might belong to another phytoplasma strain or group. Continued effort is necessary in order to identify the parthenium phyllody vector(s) through a feeding test in combination with detection of phytoplasma agent from other suspected insect vectors. In fact, the detection of phytoplasma in an insect does not necessarily mean that the insect species can transmit the disease (Vega et al. 1993). Hence, PCR with FBP group specific primers and/or RFLP analysis of the PCR amplified products with different restriction enzymes coupled with feeding test may be necessary. Knowledge of the insect vector(s) that transmit phyllody disease is important for future application of phyllody disease as a biocontrol of parthenium. In areas where the disease is not prevalent, crop/plant growers can be advised and/or supported to use insect vectors if the risks of specificity phyllody phytoplasma and vector (s) transmitting the agent are found safe to economically important plants growing in the country.

During the survey work, it was observed that peanut witches'-broom/phyllody syndrome is heavily infecting peanut in the Melka Werer area, though the disease aetiology is not yet known (personal observation). Similarly, there are reports of faba bean (*Vicia faba*) phyllody (Dereje and Tesfaye 1994) and sesame (*Sesamum indicum*) phyllody in Ethiopia (Adane Abreham, personal communication). These plants were found in the same areas where the diseased parthenium plants were collected. That may lead to the assumption one can assume that parthenium phyllody disease might have originated from its collateral hosts. Mathur and Muniyappa (1993) carried out host range studies in India with 16 plant species belonging to Asteraceae, Fabaceae, Malvaceae, Pedaliaceae and Solanaceae by inoculating the plants using leafhopper, *Orocius albicinctus,* as a vector. They reported that parthenium phyllody agent was transmitted to parthenium (96%), sunnhemp (100%), aster (66%), blackgram (7%), cowpea (5%), field bean (25%), greengram (10%), lupin (20%), horsegram (10%), pigeonpea (8%), sesame (70%), and soybean (20%). This suggests that parthenium phyllody attacks a wide variety of crops and that a host-specific study is imperative before its application for biological control.

From the current and other studies, it is evident that PCR provided a sensitive and rapid means of confirming phytoplasma infection. Such an assay can now be used to determine the host range and distribution of phyllody phytoplasma on economic crops and related weeds, in Ethiopia. Therefore, further research is needed 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 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 can, therefore, be utilised as a classical biological control of parthenium by inducing phyllody using vectors of the pathogen responsible for the disease in order to check its growth and reduce its competitiveness. Healthy parthenium plants in fallow lands, roadsides and grasslands might be controlled by releasing the leafhopper vector(s). However, host range studies of phyllody disease in relation to economic plants is needed to determine the potential risk. In addition, confirmation of insect vectors that transmit phyllody for utilisation in areas where the disease is not present in Ethiopia is imperative.

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