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# *In vitro* Detection of Yeast-Like and Mycelial Colonies of *Ustilago scitaminea* in Tissue-Cultured Plantlets of Sugarcane Using Polymerase Chain Reaction

S. Ali Moosawi-Jorf and Mahin B. Izadi
Department of Plant Protection, Shahid Chamran University, Ahwaz, Iran

**Abstract:** Plantlets of sugarcane cultivars NCO-310 (susceptible) and CP73-21 (resistant) were generated using *in vitro* apical meristem tissue culture method of leaf and culturing of the callous. Yeast-like and dikaryotic mycelial colonies were isolated and purified. The plantlets were inoculated with two types of yeast-like and dikaryotic mycelial colonies. Results of the PCR assay in plantlets inoculated with the two types of colonies indicated the detection of bE mating-type gene of sugarcane smut in all treated plantlets at all different times after inoculation. Whereas, the disease symptoms were seen in cuttings inoculated only with dikaryotic mycelia or mixed mating types of sporidia, 6 month after transplanting in pots.

**Key words:** Sugarcane, smut, *Ustilago scitaminea*, PCR, detection, tissue culture

## INTRODUCTION

Sugarcane smut was first reported from Natal in South Africa (McMartin, 1945). After that, the disease was observed in most of sugarcane-producing areas of the word and in several areas, severe epidemics have occurred (Ferreria and Comstock, 1980). In Iran, the disease was reported in 1971 from Haft-Tappeh and it had some problems in sugarcane production (Ershad and Bani-Abbasi, 1971; Moosawi-Jorf *et al*., 2006). Now the disease occurs in all sugarcane-producing countries except Papua New Guinea (Singh *et al*., 2004).

Teliospores of *Ustilago scitaminea* germinate and produce small hyphae called promycelium. Each cell of promycellium produces a sporidium (Alexander and Krishna, 1978). Germination of sporidia leads to produce germ tube or same sporidial cells (Chona, 1943). Infective hypha penetrate from lower parts of buds and below the scales. This hypha establish in apical meristem in short time as dormant hypha up to symptom production (Sinha, 1982; Agnitori, 1990). Each infected bud is a distinct infective unit and the fungus can distribute from an infected bud to other parts (Ricaud *et al*., 1989).

Recently, the Polymerase Chain Reaction (PCR) technique is used for recognition, identification and detection of different plant diseases because of its sensitivity, carefulness and high speed. Besides, in comparison with classic ratoon multiplication of sugarcane, using the tissue culture method can produce many plantlets in short time, so these plantlets may be used in pathogenicity test in controlled environments for prevention of probable dispersal of teliospores in fields.

The objective of this study was to detect yeast and mycelial spread of the fungus in different parts of inoculated tissue cultured plantlets of sugarcane using PCR method.

## MATERIALS AND METHODS

**Preparation of tissue-cultured plantlets:** The leaf-roll culture method was used to generate 5-7 cm tall of plantlets of sugarcane cultivars NCO-310 (susceptible) and CP73-21 (resistant) by callus propagation *in vitro* (Singh *et al*., 2004) in Sugarcane Research Institute, Ahwaz, Iran at 2005-2006. To do so, middle parts of leaf-roll around the apical meristem were prepared. These specimens were surface disinfected in $HgCl_2$ solution (0.7 g $HgCl_2$ $L^{-1}$ sterile distilled water) for 15 min, then divided into disks of 5-7 mm in diameters. Disks were transplanted in plates containing MS medium amended with 2-3 mg $L^{-1}$ 2,4-D. Plates were incubated in dark at 25°C. For regeneration of prepared callous, the callous was divided in pieces of 3-6 mm in diameters and transplanted in plates containing MS medium amended with 1 mg $L^{-1}$ BAP hormone (Tylor and Dukic, 1993). Plates were incubated in continuous white light at 25°C. Plantlets were grown from regenerated callous. Therefore, the regenerated callous were transferred in 25 mL glass tubes containing MS media with 1 mg $L^{-1}$ BAP hormone and incubated in growth chamber at 25°C and continuous

**Corresponding Author:** S. Ali Moosawi-Jorf, Department of Plant Protection, Shahid Chamran University, Ahwaz, Iran

white light. Callous mass with 2-5 mm of plantlets were transferred to the same culture and the same conditions.

**Preparation of inoculum**

**Monosporidial cultures:** Isolation and purification of the *U. scitaminea* took place by culturing on YGC medium. Teliospores were collected from whips of infected sugarcane in Khuzestan Province, Iran. Teliospores were surface disinfected in sterile distilled water amended with 0.3 mg $L^{-1}$ streptomycin sulfate for 2 h, then the teliospore suspension were streaked on YGC culture medium using sterile loop. Plates were incubated in dark for 24 h at 28-30°C. Pure cultures of sporidia initiated from teliospores. For preparation of monosporidial cultures, the 24 h old cultures of germinated teliospores were used. To do so, fine diluted suspension of sporidia were prepared from germinated teliospores on YGC medium. Suspension were spread on YGC and incubated in dark for 24 h at 28-30°C. Then each yeast-like monocolonies that produced on the culture, were streaked again by loop on YGC and plates incubated in dark for 48 at 28-30°C. To determine the mating type of pure monosporidial cultures, cream yeast-like colonies from seven days old culture media of monosporidia were produced on YGC. Mating types of the monosporidial culture were determined as – or + by cross-streaking them on medium. All possible pair combinations of monosporidial cultures were crossed on medium. White floccose erected mycelia were produced in compatible crosses (that has been postulated to include different mating types), whereas in incompatible crosses (that has been postulated to include same mating type), the colonies were survived yeast like. Different mating types were randomly postulated – and +.

To preparation of monosporidial inoculum suspension, surface of 2-3 days old of monosporidial cultures containing yeast-like colonies of each different mating type were crashed by added 1 mL of sterile distilled water. The suspension was transferred to sterile vials. Concentration was adjusted at $10^6$-$10^7$ sporidium per mL using homocitometer. These suspensions were maintained on ice and used as inoculum up to 4 h after preparation.

**Dikaryotic mycelium:** To produce the dikaryotic mycelia of the fungus, mixture of – and + sporidial suspension were spread on YGC medium. Plates were incubated in dark for 48 h at 28-30°C. So, dikaryotic mycelia initiated from a mixture of + and sporidia were obtained on YGC culture medium. A disc 1 cm in diameter of one week old of dikaryotic mycelial growth was transferred to vials containing 1 mL of sterile distilled water and vortexed. These suspensions were maintained on ice and used as inoculum up to 4 h after preparation.

An alternative method was used for producing the dikaryotic mycelia. In this method, the fungal mycelia were isolated from infected plant tissues including whips. To do so, pieces of stems from 2-5 cm below the whips were collected. Pieces were surface disinfected with 0.05% sodium hypochlorite, rinsed with sterile distilled water three times and plated into the PDA amended with 250 mg $L^{-1}$ streptomycin sulfate. Plates were incubated in dark at 28-30°C and surveyed for dikaryotic mycelia daily. So, dikaryotic mycelia initiated from infected plant tissue were obtained on PDA culture medium. A disc 1 cm in diameter of 3 weeks old of dikaryotic mycelial growth was transferred to vials containing 1 mL of sterile distilled water and vortexed. These suspensions were maintained on ice and used as inoculum up to 4 h after preparation.

**Inoculation of tissue-cultured plantlets and cuttings:** Plantlets of each sugarcane cultivar NCO-310 (susceptible) and CP73-21 (resistant) were inoculated at above the apical meristem, by cutting of 3-5 mm from apical meristem and placement of 7 μL of a suspension containing $1\times10^6$ sporidia per mL of 5 kinds of inoculums including either + or – mating type; or a 1:1 mixture (plus and minus) mating types; or blend dikaryotic mycelium initiated from a mixture of + and – sporidia and/or from infected plant tissue, using a Hamilton syringe. Cuttings of the cultivars were injected at below of swelled scales of buds with same inoculums. Check plants were inoculated with the same amount of sterile distilled water. Smut-inoculated and control plantlets included offshoot that not inoculated. Plantlets were transferred in 25 mL glass tubes containing MS media without hormones and incubated in growth chamber at 25°C and 18 h photoperiod. Inoculated cuttings were transplanted in pots and incubated in greenhouse at 28-32°C for 6 months.

Plantlets were divided into 3 zones a, b and c. Zone a represents the portion of culms above the point of inoculation. Zone b was the portion of the culms below the point of inoculation and Zone c represents the basal portion of the offshoot. Samples were harvested on the 18 h, 3 days, 1, 2 weeks and 1 month after inoculation for DNA extraction.

**Polymerase Chain Reaction (PCR) amplification:** DNA was extracted from pieces of smut-inoculated and checked plantlets of sugarcane with offshoot following the method of Murray and Thampson (1980).

PCR amplification was carried out in a total volume of 25 μL containing 2 mL of extracted DNA, 0.24 μM each of the specific primers bE4 (5'-CgCTCTggTTCATCAACg-3') and bE8 (5'-TgCTgTCgATggAAggTgT-3') which

amplified the mating type gene of *U. scitaminea* (Albert and Schench, 1996). The reaction mixture also contained 0.2 mM dNTPs, 0.5 mM $MgCl_2$ and 1.5 unit $\mu L^{-1}$ *Taq* DNA polymerase.

The reaction was run for 30 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec and extension at 72°C for 1 min. Finally, 7 μL of each PCR product was analyzed by electrophoresis. For positive and negative check, DNA from check plantlets and sterile water were used.

## RESULTS

**Tissue culture:** Transplantation of specimens to the MS basal medium amended with 2, 4-D induced white to cream callous production after 2-4 weeks. The callous mass was multilayered in the same medium after more 2-4 weeks. Transfer of callous mass to MS basal medium amended with 1 mg $L^{-1}$ BAP hormone for 2-4 weeks was induced to regenerate and differentiate callous tissues and greening of them.

**Isolation and purification of yeast-like and mycelial colonies:** Teliospores began to germinate 20 min after culture. Often teliospores were germinated on YGC medium after 24 h and produced promycelia and sporidia (Fig. 1) or produced mycelia. Forty eight hours after spreading the germinated teliospores on YGC, yeast-like and white floccose erected mycelial colonies were produced as mixture colonies on medium (Fig. 2).

Monosporidial cultures were obtained by streaking of yeast-like colonies on YGC medium after 48 h at 28°C (Fig. 3). Cross-streaking of positive and negative mating types of monosporidial sporidia were leaded to form the white floccose erected mycelial dikaryon at the point where they cross on culture medium after 48 h (Fig. 4).

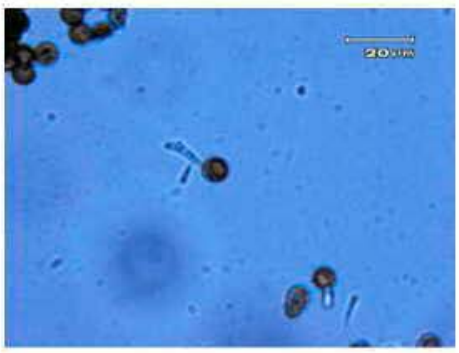

Fig. 1: Teliospore germination of *Ustilago scitaminea* and production of promycelium and sporidia

White mycelial colonies were grown on PDA, 5 days after culturing of pieces of stems from infected plants. White hyphae were uniformly spread on and around all cultured pieces (Fig. 5).

**Disease symptoms in inoculated cuttings:** Infection was seen only in cultivar NCO-310 (susceptible) inoculated by

Fig. 2: Yeast-like and mycelial colonies of *Ustilago scitaminea*

Fig. 3: Yeast-like colonies of *Ustilago scitaminea*, produced monosporidia

Fig. 4: White floccose erected mycelia of *Ustilago scitaminea* at the point where the − and + mating type were crossed on culture medium

Table 1: Infection percents in sugarcane cultivars NCO-310 (susceptible) and CP73-21 (resistant) inoculated with 5 kinds of inoculums

| | Kind of inoculum | | | | |
|---|---|---|---|---|---|
| Cultivars | + mating type | – mating type | a 1:1 mixture (plus and minus) mating types | Blend dikaryotic mycelium initiated from a mixture of + and – sporidia | Blend dikaryotic mycelium initiated from infected plant tissue |
| NCO-310 (susceptible) | 0 | 0 | 33 | 28 | 31 |
| CP73-21 (resistant) | 0 | 0 | 0 | 0 | 0 |

Fig. 5: White mycelial growth of *Ustilago scitaminea* from infected pieces of sugarcane

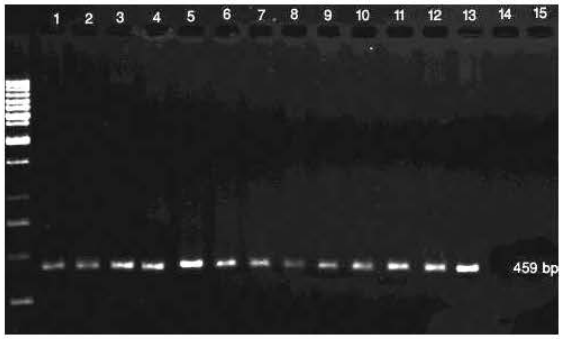


Fig. 6: Amplification of 459 bp fragment from sugarcane smut in DNA extracted from plantlets inoculated with sporidia. (1: Zones a, in NCO-310 (susceptible) inoculated with – /+ mating types; 2: Zone a, in NCO-310 (susceptible) inoculated with a 1:1 mixture (plus and minus) mating types; 3: Zone b, in NCO-310 (susceptible) inoculated with –/+ mating types; 4: Zone b, in NCO-310 (susceptible) inoculated with a 1:1 mixture (plus and minus) mating types; 5: Zone c, in NCO-310 (susceptible) inoculated with –/+ mating types; 6: Zone c, in NCO-310 (susceptible) inoculated with a 1:1 mixture (plus and minus) mating types; 7: Zones a, in CP73-21 (resistant) inoculated with – /+ mating types; 8: Zone a, in CP73-21 (resistant) inoculated with a 1:1 mixture (plus and minus) mating types; 9: Zone b, in CP73-21 (resistant) inoculated with –/+ mating types; 10: Zone b, in CP73-21 (resistant) inoculated with a 1:1 mixture (plus and minus) mating types; 11: Zone c, in CP73-21 (resistant) inoculated with –/+ mating types; 12: Zone c, in CP73-21 (resistant) inoculated with a 1:1 mixture (plus and minus) mating types; 13: DNA extracted from sporidia; 14: DNA extracted from checked plantlets inoculated with sterile distilled water; 15: Sterile distilled water), Zone a representing the portion of culms above the point of inoculation, Zone b representing the portion of culms below the point of inoculation and Zone c representing the basal portion of the offshoot

a 1:1 mixture (plus and minus) mating types; or blend dikaryotic mycelium initiated from a mixture of + and – sporidia and/or from infected plant tissue. No infection was observed in cultivar NCO-310 (susceptible) inoculated by either + or – mating types. Cultivar CP73-21 (resistant) was not shown any infection using 5 kinds on inoculation methods. Infection percentage in each treatment inoculated with different inoculum was shown in Table 1.

**Smut detection by PCR**

**Specificity of PCR reaction:** To detect the presence of the smut in samples of different treatment by PCR; specific primers bE4 and bE8 were used. The primers specifically amplified and detected a DNA fragment of 459 bp at in annealing temperature of 57°C in all the treatments of the plantlets inoculated with the fungus as well as in the DNA extracted from sporidial cultures *in vitro*. This DNA fragment was not detected in plantlets inoculated with water and purred water as checked specimens. The PCR assay yielded a positive response for any part (a, b and c) of the plantlets tested in all the sampling times from 18 h to one month post inoculation. Results of the PCR assay confirmed the presence of the pathogen in all parts of the inoculated plantlets.

**Smut detection by PCR in inoculated plantlets:** The PCR assay for DNA extracted from inoculated plantlets by sporidial suspension, either + or – mating type; or a 1:1

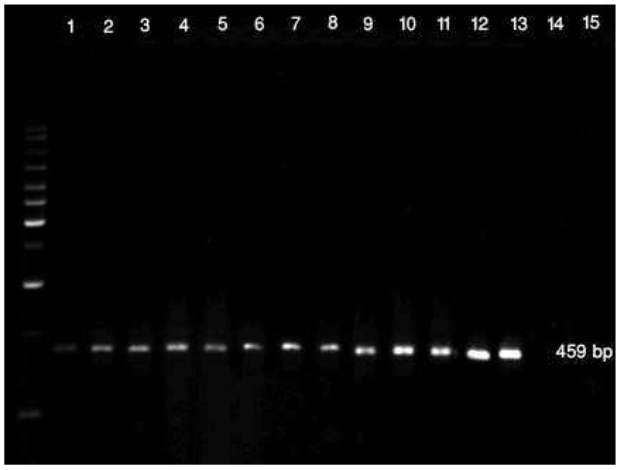


Fig. 7: Amplification of 459 bp fragment from sugarcane smut in DNA extracted from plantlets inoculated with dikaryotic mycelia (1: Zone a, in NCO-310 (susceptible) inoculated with blend dikaryotic mycelium initiated from infected plant tissue; 2: Zone a, in NCO-310 (susceptible) inoculated with blend dikaryotic mycelium initiated from a mixture of + and-sporidia; 3: Zone b, in NCO-310 (susceptible) inoculated with blend dikaryotic mycelium initiated from infected plant tissue; 4: Zone b, in NCO-310 (susceptible) inoculated with blend dikaryotic mycelium initiated from a mixture of + and-sporidia; 5: Zone c, in NCO-310 (susceptible) inoculated with blend dikaryotic mycelium initiated from infected plant tissue; 6: Zone c, in NCO-310 (susceptible) inoculated with blend dikaryotic mycelium initiated from a mixture of + and-sporidia; 7: Zone a, in CP73-21 (resistant) inoculated blend dikaryotic mycelium initiated from infected plant tissue; 8: Zone a, in CP73-21 (resistant) inoculated with blend dikaryotic mycelium initiated from a mixture of + and-sporidia; 9: Zone b, in CP73-21 (resistant) inoculated with blend dikaryotic mycelium initiated from infected plant tissue; 10: Zone b, in CP73-21 (resistant) inoculated with blend dikaryotic mycelium initiated from a mixture of + and − sporidia; 11: Zone c, in CP73-21 (resistant) inoculated with blend dikaryotic mycelium initiated from infected plant tissue; 12: Zone c, in CP73-21 (resistant) inoculated with blend dikaryotic mycelium initiated from a mixture of + and − sporidia; 13: DNA extracted from sporidia; 14: DNA extracted from checked plantlets inoculated with sterile distilled water; 15: Sterile distilled water), Zone a representing the portion of culms above the point of inoculation, Zone b representing the portion of culms below the point of inoculation and Zone c representing the basal portion of the offshoot

mixture (plus and minus) mating types in all sampling time of 18 h, 3 days, 1, 2 weeks and 1 month after inoculation, specifically yielded a positive response to amplify of primers bE4 and bE8 for any part (a, b and c) of the plantlets (Fig. 6).

In this research, tissue-cultured plantlets were also inoculated with dikaryotic mycelia initiated from a mixture of + and - sporidia and/or from infected plant tissue. The PCR assay for DNA extracted from plantlets inoculated by dikaryotic mycelia in all sampling time of 18 h, 3 days, 1, 2 weeks and 1 month after inoculation, specifically yielded a positive response to amplify of primers bE4 and bE8 for any part (a-d) of the plantlets (Fig. 7).

DNA extracted from all parts of checked plantlets inoculated with sterile distilled water did not yield any amplification products under similar PCR conditions. Whereas, primers bE4 and bE8 of *U. scitaminea* specifically amplified a DNA fragment of 459 bp at an annealing temperature of 57°C in all the treatments of the plantlets inoculated with the fungus sampled from all zones including the portions of culms below the point of inoculation (zone a), the portion of the culms above the point of inoculation (zone b) and the basal portion of the offshoot (zone c), as well as in the DNA extracted from sporidial cultures *in vitro*.

## DISCUSSION

In field and Laboratory conditions, detection of sugarcane smut fungus at early stages of plant colonization by fungus using microscopic studies is very difficult. Since these hypha can not morphologically recognize from other fungal hypha in plant infected tissues. So, microscopic detection is not exact technique

for recognition of smut in infected plant as a quick method (Lioyd and Pilly, 1981). Whereas, the production of sori occurs between 6 and 12 weeks after inoculation, so a quicker method for detection of the fungus such as PCR assay seems is needed. In comparison with methods based on microscopic observation, based-PCR molecular methods using specific primers can sensitively and specifically detect the presence of small quantities of smut DNA mixed with sugarcane DNA (Schenck, 1998).

In this investigation, specific primers were used for detection of smut fungus in sugarcane plantlets. The PCR assay detected smut DNA in all inoculated plantlets parts as well as in non-inoculated offshoots of same plantlets.

Albert and Schenck (1996) amplified a homolog of bE mating-type gene of sugarcane smut using PCR assay. These primers sensitively detected the presence of *U. scitaminea* DNA. Also, Singh *et al.* (2004) using of this specific primers detected sugarcane smut DNA in all susceptible and resistant plant parts inoculated with sporidia, but their microscopic observations were failed or difficult to detect the smut in plant even one week after inoculation (Singh *et al.*, 2004). In this study, sugarcane smut was detected in inoculated susceptible and resistant plantlets as well as in non-inoculated offshoots of same plantlets. As regards the results of smut detection in offshoots of inoculated plantlets 18 h after inoculation using PCR assay in this study, difficulty of the fungus detection in tissue plant using microscopic observation and extension of hyphal growth around the vascular tissues (Singh *et al.*, 2004), there is the possibility that *U. scitaminea* spread quickly and systemically in plant through vascular system. In this study, inoculation was done with sporidial inoculum as well as dikaryotic mycelial inoculum. In former inoculation method with dikaryotic mycelia, sugarcane smut was also detected in susceptible and resistant plantlets using PCR assay. According to Agnitori (1990), three kinds of colonies including yeast-like, white and black mycelia were isolated from infected plant. According to smut detection in inoculated plantlets and non-inoculated offshoots of same plantlets with yeast-like and mycelial inoculums of this study using PCR, it was indicated that sugarcane smut probably spread as yeast-like and mycelial form in infected plant systemically.

Results of cutting inoculations and detection of fungus in plantlets using PCR reaction in this study indicated that, regardless of existence of the smut fungus in plants, infection was produced only with dikaryotic forms.

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