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NEW HOST RECORD OF *FUSARIUM OXYSPORUM* SCHLECHT., IN PAKISTAN CAUSING WILT OF STRAWBERRY (*FRAGARIA ANANASSA* DUCH.), AND *IN VITRO* CONTROL

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ABSTRACT

The study was conducted on a common complaint of strawberry farmers regarding stunted growth and death of strawberry runner saplings. Of the 14 symptomatic samples, 9 were recovered with *Fusarium oxysporum* isolates, 03 isolates of *Pythium aphanidermatum* (Edson) Fitzp., and 02 isolates of *Fusarium solani* (Mart.) Sacc., from the diseased strawberry roots on mycological media. The pathogenicity test confirmed *F. oxysporum* isolates as highly virulent in screen house conditions. The infected plant growth was retorted and finally died ironic to control treatments. The strains of *F. Solani* and *P. aphanidermatum* showed a-pathogenic effect on the host when bio assayed @ $1.0X10^7$ spores added to the plant soil. The *F. oxysporum* strains were treated *in vitro* with fungicide concentrations. The fungicide Carbendazim showed the lowest LC_{50} value (4.5 cm diameter radius inhibition zone) 0.032 to 0.036 ppm and Difenoconazole showed 0.04 to 0.052 ppm against the spores germination on poised mycological medium. Carbendazim and Difenoconazole were also efficient to restrict mycelia growth on the poised mycological media with LC_{50} ranges from 5.4-7.9 and 5.7-13.8 ppm, respectively.

Key-words: *Fusarium oxysporum*, strawberry, new host, Pakistan, chemical control.

INTRODUCTION

The history of strawberry plant cultivation is very recent in Khairpur district, Pakistan (Qureshi *et al*., 2013). The plant nurseries (runners) are almost exotic to the district and even to the country. There are several soil borne fungal pathogens of strawberry plant *viz*. *Verticillium dahliae* (Thomas, 1932), *Phytophthora fragariae* (Wilcox *et al*., 1993), *Fusarium* spp., *F. oxysporum* (Koike, *et al.*, 2009), *Rhizoctonia fragariae* (Hussain and McKeen, 1963), *R. solani* (Fang *et al*., 2013) and *Pythium* spp. (Martin and Loper, 1999).

The *F. oxysporum* is potential pathogen causing foliage wilting, drying and withering of older leaves, stunting of plants, reduced fruit production, yellow brown discoloration of the plant crown. Plants eventually collapsed and die. Internal vascular and cortical tissues of plant crowns showed a brown-to-orange-brown discoloration, the symptoms also include black root rot (Koike *et al*., 2009).

The study was carried out on the common strawberry wilting reports of the local farmers and samples were examined under laboratory conditions for recovery of potential pathogen following all the symptoms of the diseased strawberry plants.

MATERIALS AND METHODS

Collection of samples: The different strawberry farms were visited and the diseased samples were collected and brought to the laboratory. The samples were packed in sterile polyethylene bags and placed in ice boxes. The sample were given laboratory accession number and placed at 4 ºC for < 5 days. The plant samples were preserved in FAA preservative solution in glass jars with tight lid, after inoculation of diseased tissue on the fungal culture media.

Isolation of fungi: The root of the plant was cut into pieces of 2 cm size with sterile dissection blade. The crown of the plant was cut into equal halve and the central tissue of 2 cm^2 was taken. Each root and crown part rinsed with sterilized distilled water and surface sterilized in 01% sodium hypochlorite aqueous solution for 6 minutes and again rinsed with sterile water. The surface sterilized plant tissue/root was dried with sterile blotting paper and inoculated at specialized mycological media, viz. Potato Dextrose Agar (PDA) containing agar 16g, Dextrose 20g, Potato extract of 250g with amendment of 100 ppm streptomycin and 250 ppm Penicillin antibiotics L^{-1}. The PDA and the

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V8 juice agar media (medium containing the extract of 200 g vegetables and 1.6% agar L^{-1} added with Nystatin 25 ppm and Ampicillin 500 ppm) were used for the recovery of mitosporic and Oomycetes fungi, respectively.

The inoculated plates were incubated at 20 ºC, dark until the conspicuous growth on the tissue were observed. The fungus was pure cultured through transfer of hypha at respective media, aseptically. The successive pure culture colony was short term stored at PDA poured slants for 30 days and the culture was stored in glycerol containing 5% casein in vials at - 4 ºC for long term storage.

The fungi were identified on the basis of morphological characters (Colony, Conidia, Oogonia, Sporangia, anthredia, Phialides and their size, shape) with reference to Gerlarch and Nirenberg (1982), Watanabe (2010), Barnet and Hunter (1998), Domsch *et al*. (1980) and Dick (1990).

Pathogenicity test: The host plants were grown in (3:1, loamy soil: cow dung) soil. The soil was mixed and sundried and packed into plastic bags (30 x 30 cm) after addition of 2 drops of formaldehyde for sterilization for 24 h and moistened with sterilized water. The pots were left over night in screen house and disease free, healthy strawberry runners were transplanted into the pots. The plants were irrigated every day with tap water for moderate moisture contents and fertilized with 01g of DAP after 7 days, in soil. The fungal spores were harvested in 0.2% Tween20 aqueous solution and counted by using Haemocytometer and spore concentration adjusted to $1.0x10^7$ spores mL^{-1}. There were 15 treatments with three replicates. Another set of three potted plants was treated with 0.2% aqueous solution of Tween 20 only for control treatments. The treatments were observed against the defined symptoms of wilt, stunted growth and dying. The diseased plants were uprooted and their roots and crown parts were inoculated on mycological media for recovery of the respective pathogen. The symptoms and recovery of the fungal isolate in treated plant was the confirmation of pathogenicity.

***In vitro* efficacy of fungicides to pathogenic isolate:** The pathogenic fungal spores $1.2x10^7$ mL^{-1} were mixed in pre-poured PDA medium when the medium cooled prior to solidification after autoclave sterilization. The standard discs of fungicides were placed at the centre of poured plates. The spore germination on the medium and inhibition zone around the fungicide disc was measured after 24 h. The fungicide Carbendazim and Difenaconazole were used in this study; the disc standards were of 100, 50, 25 and 10 µg, while a fungicide free disc was used as control.

In another treatment the poised PDA media with the fungicides was also inoculated with fresh 9 mm radius fungal colony and incubated at 25 ºC for five days for calculation with radial growth of mycelia mat as compared to control colony growth placed at PDA media. The fungicides were incorporated in the PDA with the concentrations of 10, 5, 2.5 and 1.0 mg L^{-1} (ppm).

RESULTS AND DISCUSSION

There were forty six collections of suspected plants of strawberry affected by soil borne diseases. The fourteen fungal isolates were recovered from the root samples on prescribed mycological media. Of the fourteen samples nine were *Fusarium oxysporum* isolates, followed by *Pythium aphanidermatum* (three isolates) and two isolates of *F. solani* (Table 1).

F. oxysporum is important soil borne pathogen of strawberry (Aroyo *et al*., 2009; Burgess, 1981; Favel *et al.*, 2003). During field screening the stunted growth and drying of older leaves, black root and crown has given a symptomological reference to the pathogen that was observed when the fungus grew over the surface sterilized root and tissues. The micrometry of fungal conidia confirmed the *F. oxysporum* that has showed the two type of conidia present, micro and macroconidia, macroconidiophores (phialides) were not well differentiated from hyphae, macroconidia are boat shaped with slightly tapering apical cell and hooked basal cells (17-30 x 2.2-2.6µm), four ±1 celled; microconidia ellipsoidal one celled (size 5-15 x 2-3.5µm); Chlamydospores usually globose and solitary (size 5-14 µm) referred to Watanabe (2010) and Domsch *et al*. (1980).

All the isolates were tested against the healthy plants. The isolates of *F. oxysporum* were confirmed as pathogenic by causing the symptoms of wilt and black root in treated strawberry plants in pots. The pathogenic isolates FRG1, FRG2, FRG8 and FRG12 were highly pathogenic to the plant after five days of inoculations of $1x10^7$ spores per pot (Table 1; Fig. 1). The *F. solani* isolates were found avirulent to the strawberry plants, while *P. aphanidermatum* isolates FRG4 and FRG11 caused minor wilting, but the plant recovered from the symptoms. The surface sterilized roots were also not recovered with *F. solani* and *P. aphanidermatum*, while the isolates of *F. oxysporum* were recovered from diseased plant roots (Table 1).

The plants are of unknown exotic origin at district Khairpur, which are usually grown through the runners purchased from mother plants growing at Sawat, the North Western region of Pakistan. It is hypothesized that the disease has been transported with runners. It is well reported that the *Fusarium* wilt of strawberry spreads through

transplant runners of prior infected mother plants (Matuo *et al*., 1980; Nam *et al.,* 2011). It is essential to develop disease free runners from healthy mother plants, which needs a specific emphasis in disease free transplant production. The South Korean farmers have suffered from *Fusarium* wilt epidemic (Nam *et al*., 2009). Present study found a strain of oomycetes as an isolate from infected roots but the pathogenicity test proved the isolate avirulent. Similar to that was reported by Fang *et al*., (2011). They reported that the *Pythium* sp. along with *F. oxysporum* isolates were recovered from the plant roots of different regions in Western Australia, further they reported that the *Fusarium* wilt was the most abundant isolate followed by *Rhizoctonia* spp. and *Pythium* spp. from suspected plants. Present study recovered *F. oxysporum, P. aphanidermatum* and *F. solani*, while *F. oxysporum* isolates found highly pathogenic to the strawberry. There are several reports of *Fusarium* wilt from different parts of the world (Koike *et al*., 2009; Cho and Moon, 1984; Arroyo *et al*., 2009). The *Fusarium* wilt, caused by *Fusarium oxysporum* f. sp. *fragariae* Winks and Williams, is the most serious soilborne disease affecting strawberries (Nam et al., 2009), while *F. oxysporum* Schlechtend emend. Snyder & Hansen, strain was reported by Arroyo *et al*. (2009) as causal agent of strawberry wilt in Spain, on the basis of morphological studies under reference to Snyder and Hansen, (1940).

Table 1. The Isolates of *F. oxysporum* from diseased roots of strawberry plants collected from various locations and the level of pathogenicity induced by each isolate on healthy host.

Strain Code	Date of Collection	Location (GPS Co-ordinates)	Fungi	Disease intensity after time intervals (days)	
				5 days	10 days
FRG1	2-1-2015	27º30’02.51”N 68º26’12.39”E	*F. oxysporum*	+++	++++
FRG2	2-1-2015	27º29’59.73”N 68º26’10.97”E	*F. oxysporum*	+++	++++
FRG5	2-1-2015	27º31’05.15”N 68º25’47.40”E	*F. oxysporum*	+	++++
FRG7	2-1-2015	27º18’36.93”N 68º35’18.84”E	*F. oxysporum*	++	++++
FRG8	8-2-2015	27º08’32.41”N 68º29’00.70”E	*F. oxysporum*	+++	++++
FRG9	8-2-2015	27º18’52.70”N 68º36’36.84”E	*F. oxysporum*	++	++++
FRG10	5-12-2015	27º18’46.99”N 68º35’57.43”E	*F. oxysporum*	++	++++
FRG12	8-2-2015	27º08’25.22”N 68º29’03.87”E	*F. oxysporum*	+++	++++
FRG13	8-2-2015	27º08’22.75”N 68º29’04.93”E	*F. oxysporum*	++	++++
FRG3	15-2-2015	27º29’59.60”N 68º25’56.48”E	*F. solani*	–	–
FRG6	2-1-2015	27º30’01.97”N 68º26’11.84”E	*F. solani*	–	–
FRG4	2-1-2015	27º30’00.60”N 68º26’00.29”E	*P. aphanidermatum*	–	+
FRG11	15-2-2015	27º29’57.06”N 68º25’59.49”E	*P. aphanidermatum*	+	–
FRG14	2-1-2015	27º31’03.39”N 68º25’48.42”E	*P. aphanidermatum*	–	–

The marks refers as; (+) for minor wilting, (++) for wilting and yellowing of leaves, (+++) for death of leaves except apex and (++++) for complete death. The mark (–) refers the no symptom of the disease.

Table 2. The *in vitro* effect of fungicide Carbendazim on spore germination of the *F. oxysporum* isolates. The fungicide effect is represented as 50% inhibition caused by the fungi around the disc at media (LC_{50}) after three days.

Parameters	*F. oxysporum* isolates								
	FRG1	FRG2	FRG5	FRG7	FRG8	FRG9	FRG10	FRG12	FRG13
LC_{50} (ppm)	0.035	0.032	0.036	0.035	0.034	0.034	0.036	0.036	0.034
Conf. Limits	0.03-0.1	0.02-0.04	0.03-0.05	0.03-0.03	0.02-0.04	0.03-0.04	- -	0.03-0.04	0.02-0.07
Estimate-intercept	8.0-4.2	7.6-3.9	7.1-4.0	7.5-4.1	7.6-4.1	5.9-3.2	5.7-3.2	5.0-208	6.7-3.6
Chi^2 (df=21) [a]	1103.8	684.9	501.9	75.5	748.3	235.1	8342.5	153.2	905.8
p [b]	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

[a.] Statistics based on individual cases differ from statistics based on aggregated cases.
[b.] Since the significance level is less than .150, a heterogeneity factor is used in the calculation of confidence limits.

Table 3. The *in vitro* effect of fungicide Difenoconazole on spore germination of the *F. oxysporum* isolates. The fungicide effect is represented as 50% inhibition caused by the fungi around the disc at media (LC_{50}).

Parameters	*F. oxysporum* isolates								
	FRG1	FRG2	FRG5	FRG7	FRG8	FRG9	FRG10	FRG12	FRG13
LC_{50} (ppm)	0.042	0.047	0.04	0.04	0.04	0.04	0.048	0.054	0.052
Conf. Limits	0.03-0.12	0.03-0.1	0.03- 0.06	0.03- 0.04	0.03- 0.06	0.03- 0.05	--	0.04- 0.07	0.04- 0.3
Estimate-intercept	8.0-5.0	7.6-5.1	7.1-4.3	7.5-4.5	7.6-4.7	5.9-3.6	5.7-3.9	5.0- 3.7	6.7-4.8
Chi^2 (df=21) [a]	1103.8	684.9	501.9	75.5	748.3	235.1	8342.5	153.2	9.5.8
p [b]	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

[a.] Statistics based on individual cases differ from statistics based on aggregated cases.
[b.] Since the significance level is less than .150, a heterogeneity factor is used in the calculation of confidence limits.

Table 4. The *in vitro* effect of fungicides on radial mycelium growth of the *F. oxysporum* isolates. The fungicide effect is represented as 50% inhibition caused by the fungi to mycelium growth on poised media as compared to control (LC_{50}).

Fungicide	Isolate	LC_{50} (ppm)	Conf. Limits	Estimate-intercept	Chi^2 (df = 21) [a]	p [b]
Carbendazim	FRG1	0.062	0.039-0.21	1.3-1.0	79.3	0.000
	FRG2	0.059	0.036-0.27	1.2-0.9	92.06	0.000
	FRG5	0.058	0.038-0.16	1.4-1.1	83.3	0.000
	FRG7	0.059	0.043-0.11	1.4-1.0	42.5	0.004
	FRG8	0.056	0.038-0.12	1.4-1.1	67.2	0.000
	FRG9	0.05	0.034-0.11	1.5-1.0	90.9	0.000
	FRG10	0.054	0.037-0.12	1.5-1.1	80.2	0.000
	FRG12	0.079	0.05-0.20	1.0-0.9	33.5	0.000
	FRG13	0.062	0.04-0.17	1.3-1.0	65.7	0.000
Difenoconazole	FRG1	0.11	0.059-0.72	1.3-1.3	79.3	0.000
	FRG2	0.12	0.06-1.60	1.2-1.3	92.06	0.000
	FRG5	0.076	0.046-0.26	1.4-1.2	83.3	0.000
	FRG7	0.08	0.054-0.17	1.4-1.0	42.5	0.004
	FRG8	0.074	0.48-0.19	1.4-1.2	67.2	0.000
	FRG9	0.057	0.038-0.14	1.5-1.1	90.9	0.000
	FRG10	0.072	0.046-0.21	1.5-1.3	80.2	0.000
	FRG12	0.13	0.07-0.51	1.0-0.9	33.5	0.000
	FRG13	0.1	0.58-0.43	1.3-1.3	65.7	0.000

[a] Statistics based on individual cases differ from statistics based on aggregated cases.
[b] Since the significance level is less than .150, a heterogeneity factor is used in the calculation of confidence limits.

The severity of the diseased plants was sporadic in the farmers' fields which suggest the spread is in initial stage, while the continuous cropping will surely increase the intensity. The screening of the transplants and mother plants are strongly suggested for determination of disease sinks. Although, several hosts of *F. oxysporum* have been reported from the Southern Pakistan (Sindh); among these Banana (Syed *et al.,* 2015), Chilli (Mushtaq and Hashmi, 1997), Cotton (Bhatti and Somroo, 1996; Rajput *et al*., 2006), are important hosts that also suggests the pathogen is available in farm soils. Therefore, the pathogenic strain might be available at the cropping soil in Khairpur, which needs another study on distribution of the pathogen in farm soils, which is not yet been reported. Present study is the first report of *F. oxysporum* as pathogenic on strawberry crops of the district Khairpur, Pakistan.

The *in vitro* treatment of fungicides Carbendazim and Difenoconazole caused inhibition zones to the isolate strains around the standard disc on mycological media. The Carbendazim was higher spore inhibiting fungicide than Difenoconazole. The *F. oxysporum* isolates were not significantly different in the Carbendizm susceptibility readings. The 50% susceptibility (LC_{50}) was found from 0.032-0.026 ppm concentrations (Table 2, Fig. 2a).

Difenoconazole found to be less effective in spore inhibition zone than Carbendazim. The 50% zone of inhibition was recorded through probit analysis and found 4.0-5.4 ppm concentration of the fungicides (Fig. 2b).The radial growth of hyphae on the media poised with fungicides showed a decrease trend in fungicide susceptibility

than spore susceptibility. The LC_{50} values of the fungicides Carbendazim and Difenoconozole, were increased from 0.05-0.079 and 0.057-0.14 ppm against the isolates (Table 4.). The isolate FRG12 was found restricted mycelium growth with higher fungicide doses (LC_{50} 0.08-0.13 ppm) of Carbedazim and Difenoconazole, respectively.

Fig. 1. The pathogenicity caused by F. oxysporum isolate FRG1 (B, C and D), after 5 days of inoculations as compared to the healthy control plant (A).

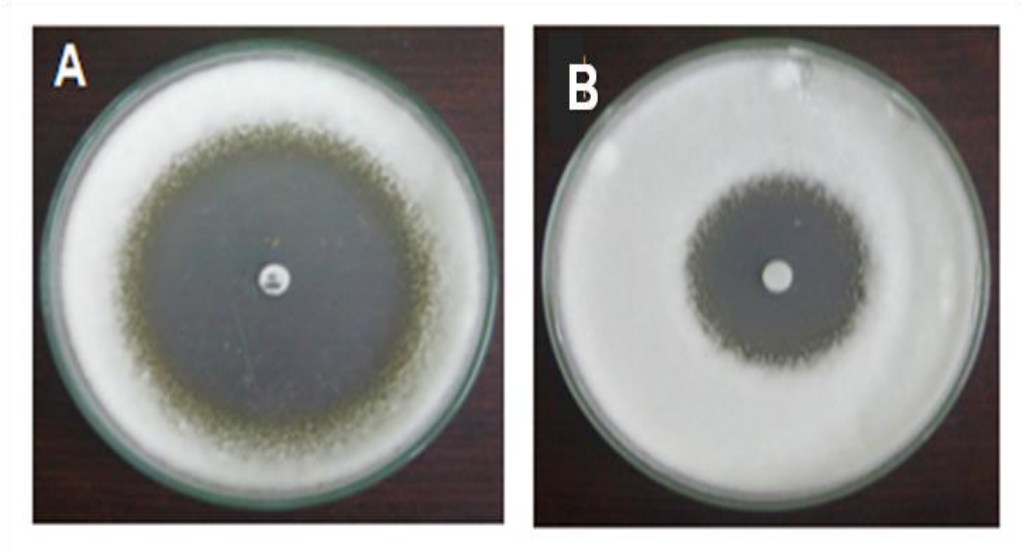

Fig. 2. The inhibition zone induced by 10 ppm dose of Carbendazim (A) and Difenoconazole (B), fungicides to the growth of spores of the F. oxysporum isolates (FRG2).

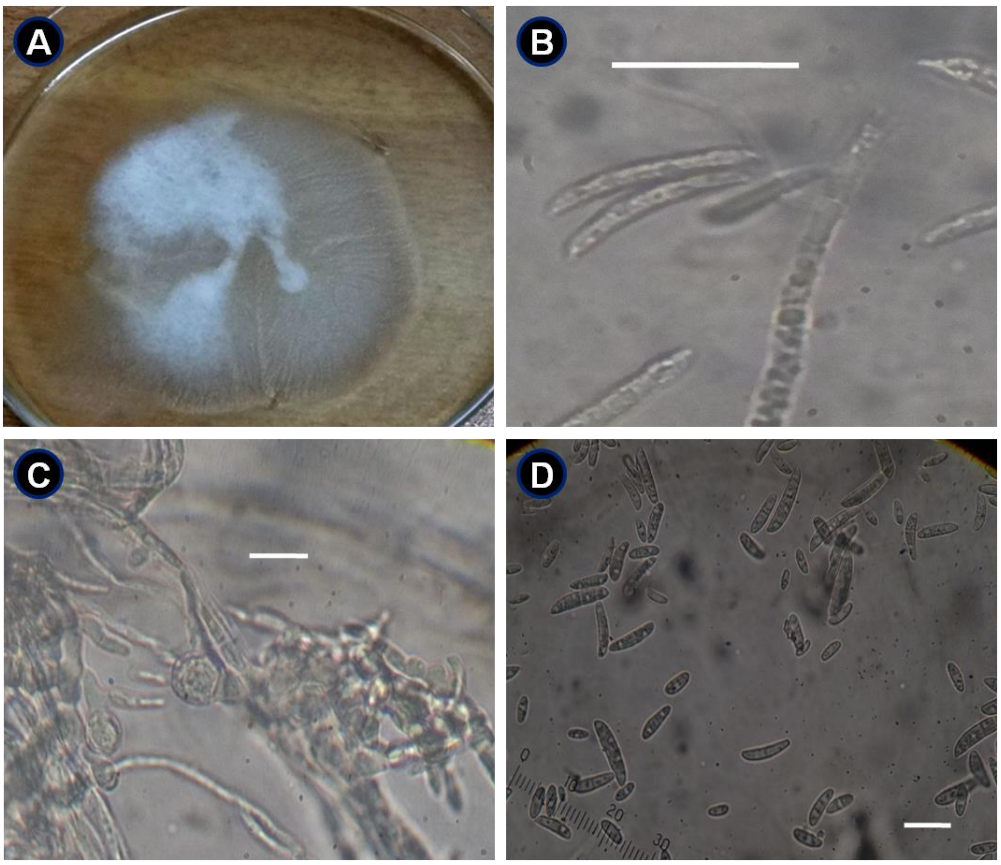

Fig. 3. *F. oxysporum* colony (A), shows the submerged growth of mycelia, the macroconidia attached with phialides of diameter less than conidia width, bar size 22µm (B), solitary chlamydospore (C) and, macro and micro conidia (D). The bars on microphotographs (C, D) refer the 12µm.

The fungicide effects were found with significant similarity with minor variations of the doses for fungal inhibition on mycological poised media, which refers the isolates are of same range of susceptibility. The hyphae inhibition showed lower susceptibility than spores, which suggest the hyphae have higher sustenance, while the hyphae needed higher doses than spores. The hyphal 50% inhibition was recorded from 0.05 to 0.08 ppm of the Carbendazim. The LC_{50} suggests the doses required to inhibit isolates are very high, which refers the isolates are of fungicide tolerance. The toxicity of Difenoconazole was reported by CaiHua (2009) to different isolates of *F. oxysporum* f. sp. *fragariae* from different farm lands of China which ranged from 0.13 to 9.0 µg/ml to cause 50% toxicity to the isolates. Amini and Sidovich (2010) recorded 0.009 ppm EC_{50} of Carbendazim to inhibit 50% radial growth against *F. oxysporum* f. sp. *lycopersici* causing tomato wilt. It is again hypothesized that the present isolates are fungicide tolerant as the farmers usually use fungicides at Khairpur and Sawat, where the strawberry transplants were grown and produced.

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