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In vitro study of the antifungal activity of saponin-rich extracts against prevalent phytopathogenic fungi

Bishnu P. Chapagain [a], Zeev Wiesman [a,*], Leah Tsror (Lahkim) [b]

[a] *Phyto-Lipid Biotechnology Laboratory, Department of Biotechnology Engineering, Institutes for Applied Research, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel*
[b] *Department of Plant Pathology, Agricultural Research Organization, Gilat Experiment Station, M.P. Negev 85280, Israel*

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Abstract

Saponin rich-extracts (SREs) from *Balanites aegyptiaca* fruit mesocarp (BE), *Quillja saponaria* bark (QE), and *Yucca schidigera* (YE) have been tested against common phytopathogenic fungi (*Pythium ultimum*, *Fusarium oxysporum*, *Alternaria solani*, *Colletotrichum coccodes*, and *Verticillium dahliae*). The inhibitory effect of these extracts was measured *in vitro* and the concentrations that will reduce the colony diameter of fungus to 50% of the control (DRC_{50} values) were determined. Dose-dependent fungal and/or saponin-specific results were obtained. The highest concentration of BE (4%) showed high (81.1%) and moderate (34.7%) growth inhibitions against *P. ultimum* and *A. solani*, respectively, whereas weak growth inhibition or weak growth stimulation occurred against *F. oxysporum*, *C. coccodes*, and *V. dahliae*. The highest concentration (4%) of QE showed moderate growth inhibition (35.9–59.1%) of all fungi except *C. coccodes*. YE showed moderate to very high (54.1–100%) growth inhibition of all fungi tested. The results of this study suggest that selected SREs can play an important role in controlling these fungi, especially under organic management. The possible structure–function relation of antifungal activities of the various SREs is discussed.
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Keywords: Saponin-rich extract; *Balanites aegyptiaca*; *Quillaja saponaria*; *Yucca schidigera*; *Pythium ultimum*; *Fusarium oxysporum*; *Alternaria solani*; *Colletotrichum coccodes*; *Verticillium dahliae*

1. Introduction

One-third of all global agricultural production is lost each year due to various pests and diseases (Bajwa et al., 2003). Among the losses, nearly 20% is the result of pathogenic fungal diseases (Agrios, 2000). Chemical compounds are the major control measure to avoid loss of yield or quality due to these fungi. No doubt the use of chemicals has been found to be effective in controlling these diseases, but some major problems threaten to limit the continued use of these chemical fungicides. One problem is the tendency of fungi to develop resistance to chemicals, necessitating a higher dose or the development of new chemicals to replace those to which fungi are resistant (Bajwa et al., 2003). Another is the creation of a hazardous environment both for human beings and other flora and fauna by these chemical fungicides because of their non-biodegradable nature (Hayes and Laws, 1991). The problem of the development of resistance may also apply to the plant produced natural compounds (Papadopoulou et al., 1999; Bouarab et al., 2002) however; such compounds always maintained ecological balance in nature. These problems highlighted

* Corresponding author. Tel.: +972 8 647 7184; fax: +972 8 647 7184.
E-mail address: wiesman@bgu.ac.il (Z. Wiesman).

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the need to develop alternative methods for controlling plant diseases; this in turn has stimulated research on the occurrence of natural botanical pesticides and the potential for commercialization of these materials (Arnason et al., 1989). Furthermore, some synthetic pesticides are currently prohibited in several countries, and others are in continuous process. In this context, the search for natural, biologically active, and renewable plant products that could replace hazardous pesticides is relevant.

Plant secondary metabolites are often rapidly degraded in soil, they generally have no mammalian toxicity, and they can have an effective role in sustainable agriculture (Saxena, 1983). Among the secondary metabolites, saponins (complex glycosidic compounds found in many higher plants) are known for their fungistatic activities (Oakenfull and Sidhu, 1989). In the plant itself, saponins are associated with the plant's defense system (Morrissey and Osbourn, 1999). Earlier studies have revealed that many saponins or saponin-rich extracts (SRE) from various plants showed antifungal activities (Oleszek et al., 1990; Hostettman and Marston, 1995; Osbourn et al., 1996). Sindambiwe et al. (1998) have reported growth inhibition of *Epidermaphyton flooccosum*, *Microides interdigitalis*, and *Trichophyton rubrum* fungi by a saponin-rich extract of *Maesa lanceolata*. Many saponins were also found to have an inhibitory effect against *Candida albicans*, *Crytococus neoformans*, and *Aspergillus fumigatus* (Li et al., 1999). Similarly, saponins from *Chenopodium quinoa*, *Capsicum annum*, *Phytolacca tetramera*, and *Panax notoginseng* were also reported to have antifungal activities (Woldemichael and Wink, 2001; Escalante et al., 2002; Iorizzi et al., 2002). However, most antifungal studies of saponins have been carried out on yeast and other fungus and a meager study has been done on pathogenic plant fungi. This encouraged us to test the effects of saponins on the growth of some common important plant pathogenic fungi. Tests of pure saponins from any particular plant on the specific pathogen is rather difficult due to necessary purification, so this study was designed to perform *in vitro* studies using only SREs, at a much lower cost.

Balanites aegyptiaca is a plant of the family Zygophyllaceae which is widely grown in the drier areas of East and West Africa, the Middle East, and southern Asia. Various parts of this plant have been used for centuries by Africans and Indians to treat a wide variety of maladies (Hall and Walker, 1991). Earlier studies revealed that *B. aegyptiaca* contains steroid saponins mainly in fruit mesocarp, roots, and bark (Liu and Nakanishi, 1982; Kamel, 1998). *Quillaja saponaria*, an evergreen tree of the Rosaceae family, is found in arid regions of Chile, Peru, and Bolivia, and contains triterpenoid saponins mainly in its bark (Cheeke, 2000). *Yucca schidigera* is a plant of the Agavaceae family which is native to the desert southwest United States, Baja California, and Mexico, whose extract contains steroidal saponins (Wang et al., 2000). Recently, the use of *Y. schidigera* has been recognized as a superior natural wetting agent and surfactant via flood or drip irrigation, and as a foliar spray to increase water and fertilizer penetration, reduce surface tension of agricultural sprays, and soften hard compacted soils (Karnan and Marx, 2000).

The objective of this paper was to test, *in vitro*, the antifungal activity of saponin-rich extracts of fruit mesocarp of *B. aegyptiaca*, bark extract of *Q. saponaria*, and plant extract of *Y. schidigera* against *P. ultimum*, *F. oxysporum*, *A. solani*, *C. coccodes*, and *V. dahliae*. This study is one of the many attempts to search for more environmentally and toxicologically safe and more selective and effective efficacious fungicides.

2. Materials and methods

2.1. Preparation of the saponin-rich extracts (SREs)

Balanites saponin extract was made by dilution of the laboratory-prepared fruit mesocarp extract of the *B. aegyptiaca*. The fruits of *B. aegyptiaca* were collected from the Balanites orchard located in Kibbutz Samar in southern Israel and authenticated by Prof. Uzi Plitman from the herbarium in the Hebrew University of Jerusalem. Voucher specimen (76816) was deposited in the herbarium of the Hebrew University of Jerusalem. The Balanites fruit mesocarp extract (BE) was prepared in our laboratory as described elsewhere (Chapagain and Wiesman, 2005). The *Q. saponin* extract (QE) was prepared by dilution of a commercial saponin extract of *Quillaja saponaria* bark purchased from Sigma (St. Louis, MO, USA). *Y. schidigera* extract (YE) was obtained from Cellu-con Inc. (Strathmore, CA, USA). The total saponin content in BE, QE, and YE was determined by measuring absorbance at 430 nm on color reaction of anisaldehyde, sulfuric acid, and ethyl acetate as described by Baccou et al. (1977) and Uematsu et al. (2000) with some modification by Chapagain and Wiesman (2005).

2.2. Fungal isolates

Cultures of *Pythium ultimum*, *Alternaria solani*, *Colletotrichum coccodes*, and *Verticillium dahliae* were isolated from diseased specimens of potato, and *Fusarium oxysporum* from jojoba, grown in the experimental

field of Gilat Station, Negev, Israel. Isolates were placed on potato dextrose agar (PDA, Difco, Detroit, MI, USA) supplemented with 100 ppm streptomycin and incubated in the dark at 27 °C. Pathogens were identified visually and microscopically, and sub-cultured on PDA without antibiotics as described by Tsror et al. (2001).

2.3. Growth inhibition measurements

Five concentrations (0.1%, 0.5%, 1.0%, 2.0%, and 4.0% (w/v)) of BE, QE, and YE were tested. A negative control (0%) and positive controls of registered fungicides were added in separate Erlenmeyer flasks containing sterilized (121 °C, 1.2 atm, 20 min) PDA and mixed properly. Samples of PDA containing BE, QE, YE, and controls (15 ml) were poured separately into sterilized Petri dishes and allowed to solidify. For the positive controls, flustriafol 100 ppm, metalaxyl 5 ppm, benomyl 5 ppm, prochloraz 100 ppm and 200 ppm were used for *A. solani*, *P. ultimum*, *F. oxysporum*, *V. dahliae*, and *C. coccodes*, respectively.

A 2 mm diameter plug of the actively growing mycelium of the fungal isolate was placed in the center of the each plate. The plates were then incubated at 27 °C, in the dark (10 plates per treatment). Measurement of the colony diameter of the radial mycelium growth was carried out according to the growth rate of each fungus. The growth of *A. solani* and *F. oxysporum* was measured on the 2nd, 4th, and 7th day. Growth measurement of *V. dahliae* and *C. coccodes* was carried out on the 3rd, 7th, and 10th day. Because of its fast growing nature, the colony diameter of *P. ultimum* was measured daily for 2 days.

For growth inhibition analysis, colony diameter data taken after 2 days (*P. ultimum*), 7 days (*A. solani* and *F. oxysporum*), and 10 days (*V. dahlia* and *C. coccodes*) were used. The inhibitory activity of each treatment was expressed as the percent growth inhibition compared to the negative control (0%) using the following formula, where DC = diameter of control, and DT = diameter of fungal colony with treatment (Pandey et al., 1982):

$$\text{growth inhibition}\,(\%) = \left[\frac{\text{DC} - \text{DT}}{\text{DC}}\right] \times 100$$

The concentration of each of the SREs that will reduce colony diameter of fungus to 50% of the control (DRC_{50} values) was determined using the Probit program. The experiments were repeated twice and the data presented here are the average of two experiments.

2.4. Statistical analysis

Statistical analysis of the data was performed with JMP software (SAS, 2000) using the Tukey–Kramer HSD test for determining significant difference among treatments at $p = 0.05$ level of significance.

3. Results

3.1. Total saponin content in the SREs

The saponin content in the three SREs used in this study, BE, QE, and YE, was found to be very similar (Table 1). The total saponins in BE, QE, and YE were 11.2%, 12.03%, and 13.5% of DW, respectively.

3.2. Effects of the SREs on in vitro growth of A. solani

The radial growth of *A. solani* was reduced by all SREs at all concentrations (Table 2), and a dose dependent effect was observed in the three extracts. The highest growth inhibition was observed with 4% YE (76.0%), followed by QE (47.5%), and BE (34.7%) at the same concentration. Two and 4% YE were significantly more effective in growth inhibition than 100 ppm flustriafol, which was used as a positive reference. In the case of BE and QE, none of the concentrations inhibited 50% fungal growth; the DRC_{50} of YE was 0.3% (Table 3).

3.3. Effects of the SREs on in vitro growth of P. ultimum

With *P. ultimum*, all SREs also showed a dose dependent effect (Table 2). The highest concentration (4%) of BE inhibited fungal growth by 81%, whereas 4% of YE and QE caused 63.4% and 59.1% growth inhibition, respectively. The inhibition by 4% BE was significantly higher than the fungicide metalaxyl at 5 ppm. The DRC_{50}

Table 1
Saponin amounts in the saponin-rich extracts (SREs)

SREs[a]	Saponins (% DW)[b]
BE	11.20 ± 0.98
QE	12.03 ± 1.04
YE	13.50 ± 1.00

[a] Fruit mesocarp extract of *Balanites aegyptiaca* (BE), bark extract of *Quillaja saponaria* (QE), and plant extract of *Yucca schidigera* (YE).
[b] Values are the mean of 10 samples ± S.E. as determined by Baccou et al. (1977) and Uematsu et al. (2000), with some modification by Chapagain and Wiesman (2005).

Table 2
Effect of saponin rich extracts (SREs) on *in vitro* mycelial colony growth of several fungi[a]

Treatments[b]	% Growth inhibition[c]				
	Alternaria solani	*Pythium ultimum*	*Fusarium oxysporum*	*Verticillium dahliae*	*Colletotrichum coccodes*
BE					
0.1	1.03 f	0.50 f	0.76 b	3.28 c	−10.21 b
0.5	8.60 e	7.85 e	0.46 b	7.54 b	−9.14 b
1.0	17.49 d	28.00 d	1.25 b	8.84 b	−8.58 b
2.0	29.16 c	70.91 c	3.28 b	9.41 b	−10.51 b
4.0	34.70 b	81.09 a	2.15 b	9.74 b	−12.69 b
Positive control[d]	65.01 a	75.05 b	56.85 a	68.11 a	95.25 a
QE					
0.1	19.70 f	26.69 f	40.81 b	17.72 c	−1.44 b
0.5	25.81 e	37.51 e	52.01 a	20.54 c	−2.44 b
1.0	34.32 d	46.32 d	53.68 a	29.35 b	−1.33 b
2.0	39.10 c	53.21 c	55.66 a	33.48 b	−0.36 b
4.0	47.50 b	59.12 b	56.11 a	35.92 b	−3.85 b
Positive control[d]	65.01 a	75.05 a	56.85 a	68.11 a	95.25 a
YE					
0.1	28.19 e	7.87 f	13.76 d	35.10 e	55.79 e
0.5	56.13 d	48.31 e	32.63 c	46.12 d	81.95 d
1.0	65.20 c	52.36 d	44.32 b	48.95 d	87.01 c
2.0	71.84 b	57.59 c	52.16 a	52.12 c	93.21 b
4.0	75.98 a	63.37 b	57.25 a	59.17 b	100.00 a
Positive control[d]	65.01 c	75.05 a	56.85 a	68.11 a	95.25 b

[a] Mesocarp extract of *Balanites aegyptiaca* (BE), bark extract of *Quillja saponaria* (QE), and plant extract of *Yucca schidigera* (YE).
[b] Each value is the mean of the 20 from two experiments ($n = 20$). Means followed by same letter in each column are not significant different at $p = 0.05$ by Tukey–Kramer HSD. % growth inhibition was calculated compared to the growth of the control (0%).
[c] % (w/v).
[d] For the positive control, flustriafol (100 ppm), metalaxyl (5 ppm), benomyl (5 ppm), and prochloraz (100 and 200 ppm) were used for *A. solani*, *P. ultimum*, *F. oxysporum*, *V. dahliae*, and *C. coccodes*, respectively.

of BE, QE, and YE was 2%, 1.6%, and 1.2%, respectively (Table 3).

3.4. Effects of the SREs on in vitro growth of F. oxysporum

Among the three SREs, BE showed no inhibitory effect against *F. oxysporum* (Table 2). QE showed fairly stable inhibition in regarding with its concentrations. The lowest concentration of QE (0.1%) showed 40.8% inhibition, whereas all other concentrations (from 0.5% to 4.0%) inhibited growth by 52.0–56.9%, which was not significantly different from the fungicide benomyl (5 ppm) used as a reference. The inhibition pattern in YE was found to be different than that of QE, where 0.1% showed only 13.8% inhibition and 4.0% showed a 57.3% inhibition. However, the inhibition by 2% and 4% YE was not significantly different than benomyl 5 ppm. None of the tested concentrations of BE inhibited 50% of the growth, but 0.6% and 2% of each QE and

Table 3
DRC_{50} values (% (w/v)) of saponin rich extracts (SREs)[a]

SREs	DRC_{50}[b]				
	A. solani	*P. ultimum*	*F. oxysporum*	*V. dahliae*	*C. coccodes*[x]
BE	>4.0	2.0	>4.0	>4.0	–
QE	>4.0	1.6	0.6	>4.0	–
YE	0.3	1.2	2.0	1.5	<0.1

[a] Mesocarp extracts of *B. aegyptiaca* (BE), bark extract of *Q. saponaria* (QE), and plant extract of *Y. schidigera* (YE) on *in vitro* growth inhibition of *A. solani*, *P. ultimum*, *F. oxysporum*, *V. dahliae*, and *C. coccodes*.
[b] DRC_{50}, the concentration that will reduce the colony diameter to 50% of the control (% (w/v)) of the SREs. The DRC_{50} value was determined using the Probit program.

YE, respectively, caused 50% growth inhibition (DRC_{50}) (Table 3).

3.5. Effects of the SREs on in vitro growth of *V. dahliae*

The effect of BE, QE, and YE on the growth of *V. dahliae* was similar to the effects on *F. oxysporum* (Table 2). BE showed a very week inhibition; 4% BE caused only 9.7% growth inhibition. QE showed a moderate growth inhibition, with 35.9% inhibition at 4%. YE at 4% inhibited 59.2% of the growth. Although there was a difference in growth inhibition effect with the three SREs, a dose dependent pattern was observed in all extracts. The results showed that none of the treatments equaled prochloraz 100 ppm which was used as the positive control. None of the concentrations of BE and QE were able to suppress 50% growth, but 50% inhibition was obtained with 2.0% YE (Table 3).

3.6. Effects of the SREs on in vitro growth of *C. coccodes*

YE showed the strongest growth inhibitory effects; 0.1% YE inhibited 55.8% growth, whereas 4.0% inhibited 100%. In contrast, slight stimulatory effects of BE and QE against the growth of this pathogen were observed (Table 2). Prochloraz (at 200 ppm), which was used as positive control, inhibited 95% of the growth and even 0.1% of YE was able to inhibit 50% growth (Table 3).

4. Discussion

Saponins are generally considered to have antifungal activities (Oleszek et al., 1990; Hostettman and Marston, 1995; Osbourn et al., 1996; Sindambiwe et al., 1998). The results of the present study agree with these earlier findings. The results show a dose-dependent fungal and/or saponin-specific response of the saponin-rich extracts against the mycelial growth of commercially important phytofungi. The dose-dependent fungal as well as saponin-specific behavior of the anti-microbial and antifungal activity of saponins has also been reported previously (Sparg et al., 2004).

The total saponin content in the three saponin rich extracts used in this study was similar (Table 1). The BE was moderately active (34.7%) against *A. solani*, but highly active (89.01%) against *P. ultimum*, showing significantly higher growth inhibition compared with the fungicide metalaxyl 5 ppm. However, for the other fungi (*F. oxysporum*, *V. dahliae*, and *C. coccodes*), BE showed either a very weak or a slight stimulatory response. QE was moderately active against *A. solani*, *P. ultimum*, *F. oxysporum*, and *V. dahliae* (47.5%, 59.12%, 56.11% and 35.92% growth inhibition, respectively), but no inhibition for *C. coccodes*. However, the growth inhibition by 0.1–4.0% QE was not significantly different than the growth inhibition obtained with the fungicide benomyl 5 ppm. YE, on the other hand, was very active against all fungi tested in this study. The YE had either moderate or high growth inhibitory effect against all fungi. Less than 0.1–2.0% of YE was found to reach 50% growth inhibition of the tested fungi (Table 3). In the case of *C. coccodes*, 100% growth inhibition occurred with the highest concentration of YE (Table 2), and even the lowest concentration (0.1%) inhibited growth by 55.8%. Two and 4% of YE significantly suppressed growth of *A. solani* compared with the fungicide used as positive control. This suggests that YE is very active against the growth of *C. coccodes*.

The fungal and/or saponin specific results achieved in this study indicate differences in the mode of action of SREs as well as the nature of the fungi. Literature review revealed that fruit mesocarp of the *B. aegyptiaca* extract contains mostly steroidal saponins (Liu and Nakanishi, 1982; Kamel, 1998; Lindin, 2002), mainly (almost 40%) bi-desmosidic furostanol steroidal saponins (M_W 1064 Da) with diosgenin as aglycone (Fig. 1A) with sugar units attached in C-3 and C-26 positions (Chapagain and Wiesman, 2006). The main saponin constituents of the QE are triterpenoid saponins with quallic acid as aglycone (Fig. 1B) (Hostettman and Marston, 1995). YE contains both spirostanol and furostanol glycosides, but the predominant one is the spirostanol, primarily sarsasapogenin (66%) as an aglycone (Fig. 1C) (Oleszek et al., 2001). These different aglycone structures of the main saponin present in the three SREs show that the compositional differences of the saponins in the extract may play different roles in their antifungal activities. The same extract that has shown different activities with different fungi, for instance the high inhibitory activity of BE against *P. ultimum,* and slight stimulatory activity against *C. coccodes* might be related to differences in the sterol composition in the different fungus cells. The association of the saponin molecule with the sterol (mainly cholesterol) present in the fungal cell has already been reported in the literature (Morrissey and Osbourn, 1999). Alternatively the differences in detoxifying glycosidases among different fungus (Osbourn et al., 1996; Papadopoulou et al., 1999; Bouarab et al., 2002) might be the cause for the different antifungal activity of the individual saponins and/or extracts with different fungi.

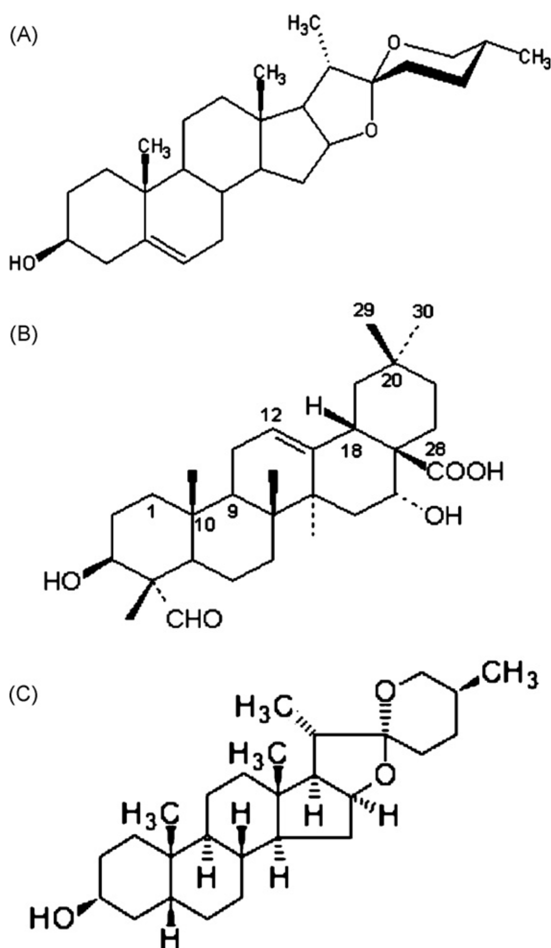

Fig. 1. Aglycone (sapogenin) structure of the main saponin of the three saponin rich extracts (SREs) used in the study. (A) Mesocarp extracts of *Balanites aegyptiaca* (BE); (B) bark extract of *Quillaja saponaria* extracts (QE); (C) plant extract of *Yucca schidegera* (YE) (Chapagain and Wiesman, 2006; Hostettman and Marston, 1995; Oleszek et al., 2001).

Extracts of *Q. saponaria* (QE) have been used for decades in diverse industries, such as food and beverages and cosmetics. The extract of *B. aegyptiaca* (BE) is popular in the traditional uses of folk remedies, particularly in the native area where this plant grows such as the Sudano-sahelian area of Africa and the Rajasthan state of India. However, the extract of *Yucca* (YE) has been used in various agricultural practices as an additive in flood irrigation to soil or in spray over foliage. It has also been reported that YE has the ability to interact with cells in the plant roots to increase water and nutrient absorption and also create a more favorable rhizosphere for the plants; this is the reason *Y. schidigera* products have been used in agriculture for years, as soil improvers, foliar sprays, wetting agents, stress control agents, and plant growth promoters, but no clear reason is available. The result of the present study may give the answer to this question.

4.1. Conclusions

Although the results obtained in this study were only from *in vitro* experiments, it has been observed that substances which are found to be fungicidal *in vitro*, in almost all cases kill the fungus *in vivo* (Kuhn and Hargreaves, 1987). The results of this study may ultimately help in the search for novel, environmental approaches to plant disease control. Saponin-rich extracts may be useful in their own right as an attractive alternative for control of fungi that attack crops, avoiding chemical fungicide applications.

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