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Formation of microsclerotia of *Verticillium dahliae* on various crops

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Abstract

In two pot experiments, potato (cvs Element, Mirka, Ostara and Astarte), pea, sugar beet, onion, flax, spring barley, field bean, spring wheat, and spring rape were inoculated with *Verticillium dahliae* by root dipping or by growing the plants in artificially infested soil. In both treatments the dry matter yield and formation of microsclerotia were determined for aerial parts. In plants grown in infested soil, the dry weight and microsclerotia formation were determined in stubbles and roots as well.

The highest numbers of microsclerotia per g plant material and per pot in the root dipping treatment were found on potato, flax and barley. The microsclerotia density on potato 'Element', pea and barley was higher in the root dipping treatment than in the soil infestation treatment. The reverse was true for potato 'Ostara' and 'Mirka'. Dry matter yield of the harvestable organs of potato 'Element' and 'Astarte', flax, sugar beet and barley was lower in the root dipping treatment than in the soil infestation treatment.

The greatest inter-crop differences in the microsclerotia yield per pot were in the aerial parts. Flax gave the highest numbers of microsclerotia per pot, followed by the four potato cultivars. The other crops had a much lower microsclerotia yield.

The results will be useful for modelling effects of various crops on the soil population at crop and farm level.

Keywords: potato, *Solanum tuberosum*, microsclerotia, reproduction, *Verticillium dahliae*

Introduction

Verticillium dahliae Kleb. was isolated by Woolliams (1966) from 51 plant species in 23 families. Its hosts include many common weeds, and they may enable the fungus to persist during rotation periods with non-hosts. Initially, *Verticillium dahliae* Kleb. was thought to be a pathogen of dicotyledonous plants. Members of the *Gramineae* were considered to be immune or non-hosts (Pegg, 1974; Schnathorst, 1981). Recommended agronomic measures to control *Verticillium* wilt have often included crop rotation with cereals. Wilhelm (1955), however, showed that *V. dahli-*

ae was present in soil even after eight years of cropping with cereals or pasture.

V. dahliae can colonise plant roots (Lacy & Horner 1966; Evans & Gleeson 1973; Malik & Milton 1980) and many authors have observed formation of microsclerotia (MS) on roots, e.g. of cereals (Harisson & Isaac, 1969). Levy & Isaac (1976) observed more extensive growth and MS were more numerous on the barley roots than on pea roots.

Krikun & Bernier (1987) isolated *V. dahliae* from leaf tissues of infected wheat, barley, oats, pea, field bean, and rape plants, and found MS in the roots of all of these crops. Symptoms and effects on yield were observed in wheat, barley, and oats, but not in pea, field bean and rape. However, Hoekstra (1989) obtained substantial evidence for reduced yield of field bean in soil infested with *V. dahliae*. Malik & Milton (1980) inoculated onion, tulip, wheat and barley plants with *V. dahliae* by root dipping or by growing the plants in artificially infested soil. During growth the treated plants remained indistinguishable from those in the non-infested control treatments, even though MS formed in the roots of the four plant species. For studies on the effect of cropping sequences on the *V. dahliae* population in soil, it is important to know to what extent the subterranean debris of each crop in a rotation contributes to the soil inoculum density.

The removal or a treatment of infected plant debris might be a useful sanitation measure. It is feasible to remove above ground plant parts, but stubble removal is more difficult, because an extra soil tillage operation is required. It is virtually impossible to remove all root debris from the soil. The ratio between the formation of MS on aerial parts, the stubble and the root system is important when assessing the effects of sanitation measures. Ben-Yephet & Szmulewich (1985) and Mol & Scholte (1995a) quantified the formation of MS in the aerial and subterranean parts of different potato cultivars in ground plant material. They found that a substantial proportion of the MS on a potato plant is formed on the subterranean parts.

In the study described here, the MS formation on aerial and subterranean parts of different mono- and dicotyledonous plant species and cultivars was quantified. To ascertain whether the method of infestation affects the infection and colonisation of plant parts by *V. dahliae*, the MS formation was assessed after the plants had been infected by root dipping or by being grown in infested soil.

Materials and methods

Experiment 1

In 1992, nine crops were grown: potato (*Solanum tuberosum* cvs Element and Mirka; the former a susceptible and sensitive cultivar, and the latter a rather resistant and tolerant cultivar (Scholte & s'Jacob, 1990)), pea (*Pisum sativum* cv. Finale), sugar beet (*Beta vulgaris* cv. Univers), onion (*Allium cepa* cv. Jumbo), flax (*Linum usitatissimum* cv. Viking), spring barley (*Hordeum vulgare* cv. Prisma), field bean (*Vicia faba* cv. Victor), and spring wheat (*Triticum aestivum* cv. Minaret). On 5 May, rooted sprouts of potato or plantlets of the other crops were inoculated by root dip-

ping and planted in 20 l pots in the open air under natural daylength and temperature (air temperatures from 5 May – 1 October 1993: mean 16.8 °C, mean minimum 11.7 °C, mean maximum 21.9 °C). The pots were dug into the field to prevent them becoming heated by solar radiation. Per pot (0.08 m^2) the plant densities were 2 for potato, 7 for pea, 1 for sugar beet, 7 for onion, 35 for flax, 15 for spring barley, 4 for field bean and 15 for spring wheat. The plants were watered by hand twice daily to avoid water stress. There were eight replications.

The following amounts of nutrients were applied per pot, apportioned over three applications from planting date to six weeks after planting: 1.8 g N, 0.5 g P, 2.7 g K, 0.2 g Mg, and 12 ml of a trace element solution containing 20 g $MnSO_4.1H_2O$, 30 g H_3BO_3, 5 g $ZnSO_4.7H_2O$, 1 g $CuSO_4.5H_2O$ and 1 g $Na_2MoO_4.2H_2O$ per litre of tap water.

Experiment 2

In 1993, the same crops as in Experiment 1 were grown, plus potato cv. Ostara (2 plants per pot) potato cv. Astarte (2 plants per pot), and spring rape (*Brassica napus* cv. Petranova; 4 plants per pot). Plantlets of each crop were planted on 28 March and grown in 20 l pots in the open air under natural daylength and temperature (air temperatures from 28 March – 1 October 1993: mean 13.8 °C, mean minimum 8.9 °C, mean maximum 18.7 °C). Each pot was wrapped in insulating foil to prevent it being heated by solar radiation. The plants were watered by hand twice daily to avoid water stress. There were six replications.

The plants were infested by root dipping or by mixing MS with the soil in which they were planted. Each experimental unit with soil mixed with MS comprised two pots: one for harvesting mature aerial plant parts and one for harvesting the roots at an early stage of growth to recover maximum amounts of roots. The soil in each pot was fertilised with the same amount of nutrients as in Experiment 1, apportioned over three applications from planting date to six weeks after planting.

Infection of plants by root dipping

An isolate of *V. dahliae* was obtained from microsclerotia naturally produced on potato cv. Bintje stems in a commercial field of the Department of Agronomy in the autumn of 1990. Pure cultures were grown on potato dextrose agar slants at a temperature of ca. 22 °C for two weeks.

Direct infestation of plants by the root dipping method was used to maximise the probability of infecting the plants. Plant seedlings or pre-rooted potato sprouts were infested by immersing their roots in a suspension of blended pure cultures of *V. dahliae* and were then planted in pots filled with a mixture of clay soil and potting compost (ratio 1:1 by volume).

Infection of plants by adding inoculum to soil

In autumn 1990, potato stems infested with microsclerotia of *V. dahliae* were collected from a commercial field of the Department of Agronomy with a history of grow-

ing potato. Stems were inspected and rejected if the sclerotia of other fungi were found. The selected stems were ground. The number of MS g^{-1} plant material was determined by image analysis (Mol & Meijer, 1995). In the experiments, the plants were grown in a mixture of a clay soil and potting compost (ratio 1:1 by volume) thoroughly mixed with MS to a final density of 30 MS per ml soil. To avoid infection at sites in the roots that had been damaged when the plants had been transplanted from the seedbed to the pots, a small hole was made in the soil in the centre of each pot and filled with non-infested soil, and the seedlings or pre-rooted potato sprouts were planted in this.

Collection of plant material

While the plants were growing, the senescent aerial plant parts were collected twice a week. The remaining aerial parts of potato, pea, flax, spring barley, field bean, spring wheat, and spring rape were harvested when the plants were mature. Aerial parts, stubble and roots were collected separately. Stubble was defined as all subterranean plant parts excluding roots and tubers but including an aerial stem base of ca 1 cm. The flax stems were harvested without separating the stubble from the stems. For the other maturely harvested crops, the aerial parts were removed 2-3 cm above the soil surface, and the stubble with the subterranean stem parts was harvested separately and washed in tap-water. Onion plants were harvested when the foliage had collapsed as a consequence of the softening of the pseudostems and when leaves started to die. As in commercial harvest, the leaves and bulbs were separated from the roots and left on the soil surface until the leaves had desiccated. Then the leaves were separated from the bulb. For sugar beet, the harvest of the leaves was in the first week of October, at the plant stage in which the sugar beet crop could be harvested commercially. The leaves and the epicotyl of sugar beet were then chopped. Since MS are formed during senescence of the host tissue, a sample of the plant material from each sugar beet pot was kept in a permeable nylon bag for four weeks on the soil surface.

Roots in Experiment 2 were harvested when the maximum root density was expected. For barley, wheat, field bean, pea, rape and flax this was at the onset of flowering, for onion at the onset of bulbing, for potato when tuber bulking started, and for sugar beet in the first half of August. For each pot, the roots were washed with tap water, and were kept for four weeks in permeable nylon bags covered with the soil they had been grown in.

After harvest and incubation, all samples were air-dried, weighed, ground, and analysed for the number of MS.

Counting the microsclerotia

In both experiments the number of MS in ground plant material was directly counted 'by eye' as described by Mol & Meijer (1995). They estimated the number of MS by counting the number of black particles after boiling small samples of ground plant material suspended in 1M sodium hydroxide.

Plant material of each sample was plated separately so that an independent test could be done for the presence of *V. dahliae*. A modified ethanol agar medium of Nadakavukaren & Horner (1959) was used. Water agar (20 g l^{-1}) was autoclaved for 20 min at 120 °C, cooled down to ca. 50 °C, and kept at 45 °C in a water bath. Just before pouring, 5 ml ethanol 96% and 50 mg chloro-oxytetracycline were added per l agar. After gently shaking, 60 plates l^{-1} were poured. Per sample, 15 mg ground plant material was spread over two plates. Uniform distribution was obtained by spreading this together with, 0.7 ml 0.1% autoclaved water-agar over the surface of the medium. The plates were incubated for 3-4 weeks in a dark room at 23 °C and a relative humidity of ca. 90%. The number of colony forming units (CFU) was counted under a stereo dissecting microscope (magnification 12x).

Results

In the plants inoculated by root dipping, large differences in MS densities were found between the experiments and among crops (Table 1). In both experiments, the highest counts were recorded for potato cv. Element. The MS densities in potato cv. Mirka, pea, flax, and barley were higher in Experiment 2 than in Experiment 1, whereas for sugar beet the opposite was found. In Experiment 2, the MS density in the four potato cultivars decreased statistically significantly from 'Element' via 'Ostara' and 'Astarte' to 'Mirka'. Flax, barley, and pea had densities more or less similar to those of 'Mirka'. The lowest numbers were found on field bean, wheat, onion, sugar beet, and rape.

Table 1. Number of microsclerotia (MS) in aerial plant material (per mg and per pot) of 12 crops inoculated by root dipping. Experiments 1 and 2.

Crop	(MS.mg^{-1})[a]		(MS per pot x10^3)[a]	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Potato 'Element'	159.3 a	121.1 a	5506 a	2372 a
Potato 'Ostara'[b]	–	59.8 b	–	953 c
Potato 'Astarte'[b]	–	39.2 c	–	799 cd
Potato 'Mirka'	7.5 bc	21.6 de	1791 b	458 de
Field bean	4.7 c	5.9 e	992 c	474 de
Pea	5.7 bc	16.5 de	401 cd	712 cd
Flax	2.9 c	27.9 cd	216 cd	2032 a
Sugar beet[c]	15.7 b	3.8 e	1800 b	95 f
Onion	7.0 bc	3.9 e	93 d	57 f
Barley	4.6 c	24.9 de	382 cd	1589 b
Wheat	2.9 c	5.0 e	504 cd	460 de
Rape[b]	–	2.4 e	–	182 ef

[a] Different letters indicate significant differences between the treatments based on LSD values (P=0.05).
[b] Not present in Experiment 1.
[c] Values are very probably overestimated because plant particles turned dark during preparation of samples.

The total number of MS per pot after infection by root dipping was calculated from the dry matter yield and the MS densities in the plant material (Table 1). In Experiment 1, potato cv. Element had by far the highest production, followed by cv. Mirka, sugar beet, field bean, wheat, pea, barley, flax and onion, in that descending order. In Experiment 2 potato cv. Element and flax had the highest production, followed by barley, potato cvs. Mirka, Ostara, and Astarte, field bean, pea, wheat, rape, sugar beet, and onion. Whereas in Experiment 2 the dry matter yields of the potato cultivars were not very different, there was a large difference in the dry matter yield between potato cvs Element and Mirka in Experiment 1. In most crops the dry matter yield was higher in Experiment 1 than in Experiment 2, causing higher MS yields per pot. In all samples, colonies of *V. dahliae* were found on the plates.

In the plants grown in infested soil, the MS density in the aerial parts and the stubble was the highest for the four potato cultivars (Table 2). The potato cultivars 'Element' and 'Ostara' had a higher MS density in the aerial parts than 'Astarte' and 'Mirka'. The MS density in the aerial parts of the other crops was much lower; the differences among crops and potato cultivars were much smaller in the stubble and root tissue than in the aerial parts. The number of MS counted in root tissue was low for all crops. In the stubble and the root very few CFU mg^{-1} were counted after plating the material. No *V. dahliae* colonies were found in the stubble of field bean and barley.

The number of MS per pot was estimated on the basis of the dry matter yield and the MS densities of the aerial parts, the stubble, and the roots (Table 3). In the aerial

Table 2. Microsclerotial density in aerial plant material, stubble and roots of 12 crops grown in infested soil and the correlation with plating on semi-selective medium. Experiment 2.

Crop	Microsclerotial formation per mg plant material		
	Aerial[a]	Stubble[a]	Root[a]
Potato 'Element'	91.2 a +++	20.2 b ++	8.9 a +
Potato 'Ostara'	83.3 a +++	29.1 a +	5.8 abc +
Potato 'Astarte'	55.7 b +++	20.2 b ++	2.5 cd +
Potato 'Mirka'	47.3 b ++	11.2 c +	5.7 abc +
Field bean	2.8 c +	1.2 d 0	1.9 cd +
Pea	3.3 c +++	3.2 d +	2.8 cd +
Flax	7.6 c +++	–[b]	6.4 ab +
Sugar beet[c]	2.8 c +	–[b]	1.1 d +
Onion	2.9 c +	–[b]	3.6 bcd +
Barley	2.2 c +++	3.4 d 0	5.3 bc +
Wheat	1.7 c +++	2.8 d +	3.6 bcd +
Rape	1.6 c ++	3.9 d ++	2.6 cd +

0 = no colony forming units (cfu), + = 1–25% cfu, ++ = 26–50% cfu, +++ = 50% cfu-all relative to the number counted by eye.

[a] Different letters indicate significant differences between the treatments based on LSD values (P=0.05)

[b] No stubble left due to the harvest methods used

[c] Values are very probably overestimated because plant particles turned dark during the preparation of the samples.

Table 3. Number of microsclerotia per pot formed on aerial plant parts, stubble or roots of 12 crops, counted by eye, after growing the plants in infested soil. Experiment 2.

Crop	Microsclerotia per pot (thousands)[a]				Rel.[b]
	Aerial	Stubble	Root	Total	
Potato 'Element'	1772 b	49.9 b	26.3 bcde	1850 b	100
Potato 'Ostara'	1452 bc	47.6 b	8.0 de	1509 bc	82
Potato 'Astarte'	1245 bc	92.2 a	5.5 e	1343 bc	73
Potato 'Mirka'	974 c	26.4 bc	13.5 cde	1014 c	55
Field bean	222 d	9.5 c	46.6 b	278 d	15
Pea	131 d	8.8 c	38.6 bcd	178 d	10
Flax	2447 a	–[c]	100.8 a	2547 a	138
Sugar beet[d]	222 d	–[c]	9.0 de	231 d	12
Onion	35 d	–[c]	14.1 bcde	49 d	3
Barley	192 d	34.2 b	117.6 a	343 d	19
Wheat	107 d	26.2 bc	40.6 bcde	174 d	9
Rape	139 d	48.3 b	43.8 bc	231 d	12

[a] Different letters indicate significant differences between the treatments based on LSD values (P=0.05).
[b] Relative differences calculated from the total production
[c] No stubble left with the harvest methods used
[d] Values are very probably overestimated because plant particles turned dark during the preparation of the samples.

parts the highest production was found in flax. Among the potato cultivars, 'Element' had a significantly higher production per pot than 'Mirka', whereas 'Ostara' and 'Astarte' were intermediate. The other crops did not differ significantly and their levels were low. Differences in the number of MS per pot were much smaller in the stubble than in the aerial parts. Potato 'Astarte' gave the highest counts, followed by potato 'Element', 'Mirka' and 'Ostara', barley, wheat and rape, whereas field bean and pea showed the lowest numbers. Per pot, barley and flax showed the highest levels of MS in the roots followed by field bean, rape, wheat, pea, and potato 'Element'. Relatively low levels were found in the roots of onion, sugar beet, and potato 'Mirka', 'Ostara' and 'Astarte'. The total number of MS per pot was the highest for flax, followed by the four potato cultivars. The other crops showed lower total levels and did not differ mutually.

The dry matter yield of harvestable organs was lower in potato 'Element' and 'Astarte', flax (seed and stem), sugar beet and barley from the root dipping treatment compared with the soil infestation treatment (Table 4). Root dipping resulted in higher levels of MS for potato 'Element', pea and barley. The difference with soil infestation was particularly large in barley. Potato 'Ostara', 'Astarte' and 'Mirka' showed statistically significantly higher levels of MS in the soil infestation treatment than in the root dipping treatment.

Table 4. Yields of harvestable organs and formation of microsclerotia on aerial debris per pot, after infecting the plants by root dipping or by infestation of the soil. Experiment 2

Crop		Yield (g dry matter per pot)		Microsclerotia per pot (x10^3)	
		Root dipping	Soil infestation	Root dipping	Soil infestation
Potato 'Element'	(tuber)	173.6**	194.7	2372*	1772
Potato 'Ostara'	(tuber)	164.2	168.2	953*	1452
Potato 'Astarte'	(tuber)	205.8**	229.7	799	1245
Potato 'Mirka'	(tuber)	227.5	225.7	458*	974
Field bean	(seed)	142.9	144.9	474	222
Pea	(seed)	74.4	67.7	712*	131
Flax	(haulm)	73.2**	139.0	2032	2447
Flax	(seed)	17.4**	36.9		
Sugar beet[b]	(beet)	237.5**	260.7	95	222
Onion[a]		–	–	57	35
Barley	(seed)	85.5*	103.7	1589**	192
Wheat	(seed)	90.6	96.2	460	107
Rape[a]		–	–	182	139

* and ** Values for yield of dry matter and microsclerotia with root dipping differ significantly from those with soil infestation at $P<0.01$, respectively

[a] No yields were measured for onion and rape

[b] Values are very probably overestimated because plant particles turned dark during the preparation of the samples.

Discussion

Methodological problems

Between the two experiments there were large differences in MS densities and total numbers of MS for some crops. This might partly be attributable to different environmental conditions in the two years, but this is unlikely to be the sole reason. Large variation in MS formation in potato between experiments has been found previously, even in experiments carried out under controlled conditions in greenhouses (Mol & Scholte, 1995a, b). Moreover, in both experiments, the differences were not similar for all crops. A difference in the time of harvest could be one of the factors.

Although colonies of *V. dahliae* were found in almost all samples after plating, there were large differences compared with the numbers counted by eye. There are at least two reasons for this. First, there is at least one MS when a CFU is counted, but there could easily be a cluster of many more MS, producing only one colony. Secondly, the germination of MS in the ground plant material could be poor, perhaps because of damage by grinding or other reasons. Also, the presence of plant tissue on the plates could have an effect: Malik & Milton (1980) found that germination of MS in tulip roots was rare when the MS were incubated in association with root tissue, but the MS germinated readily when dissected from the roots. In the current experiments, antagonistic organisms might have played a role in the low recovery after plating. This could have been a major factor in samples that were kept for some time

in the soil or on the root surface. Therefore, counting by eye with a verification for *V. dahliae* by plating seems to be the best method for quantifying MS in different crops. Another explanation for differences between the two methods is that other black particles in the samples might inadvertently be counted by eye as microsclerotia of *V. dahliae*.

Sugar beet caused the largest problems in counting MS, because many particles in the sample turned dark after boiling in sodium hydroxide. Therefore, the levels counted in the current experiments are probably overestimated. After plating, colonies of *V. dahliae* were counted. This shows that there is at least some reproduction of MS on sugar beet.

Comparison of infection methods

In plants infected by root dipping the MS density and MS yield tended to be higher than in plants infected by soil infestation. Exceptions were the potato cvs Ostara, Astarte and Mirka, and flax. A possible explanation is that the plants of the cvs Ostara and Mirka ('Astarte' showed the same tendency) were less susceptible in the early growth stage at which the roots were dipped. Their new roots grew into uninfested soil. In flax the yield was much lower after root dipping than after soil infestation, suggesting that the availability of plant tissue limits MS formation after root dipping. The dry matter yield reduction and the higher MS yield of barley after root dipping are remarkable. They confirm the findings of Mathre (1989), that wounding of barley roots facilitates infection by *V. dahliae*.

In field bean there was no yield reduction by root dipping and a very low production of MS, even though this crop is known as a good host (Hoekstra, 1989). In a field experiment, Mol *et al.* (1995) found an effect of the origin of the *V. dahliae* isolate on the yield and on the MS formation. In the current experiments, field bean may not have been sensitive or susceptible to the isolate used. Krikun & Bernier (1987) found that the colonisation of the aerial parts of gramineous crops also depended on the isolate used.

Differences among crops

The number of MS in the aerial parts was highest in potato and flax, indicating that these crops, which are known to be hosts of *V. dahliae*, were colonised systematically. In potato, the high numbers of MS were mainly caused by high MS densities, whereas in flax the high aerial dry matter yield was very important too. The higher MS formation on potato 'Element' than on 'Mirka' is consistent with previous results (Mol & Scholte, 1995a). The other crops were infected, but did not show a high density of MS in the aerial plant material.

Implications

Crops that have a high MS yield formed most of the MS in the aerial debris, which is not removed from the field with current cultivation practices. Research needs to be

done on how removing the aerial debris after harvest affects the inoculum density in the soil. This effect cannot be estimated from the data obtained in the experiments described above, because it will interact with other factors in the field (e.g. the rate at which MS are released from plant debris, mortality rate of MS). From rotational experiments with *V. dahliae* it is difficult to conclude that some crops are bad hosts. In a micro-plot experiment (Mol *et al.*, 1995), no decrease in the soil inoculum density of *V. dahliae* was found after two years of cropping with potato, pea, sugar beet, onion, flax, spring barley and field bean. This does not necessarily imply a long persistence in the soil, but might also point to a balance between formation of new MS and mortality.

Flax might contribute significantly to soil infestation if straw is left in the field after harvest. This is not common practice for fibre flax, for two main reasons: the straw is often harvested, and any straw left in the field hampers further cultivation practices. The debris of the other crops is usually left in the field. Once a good method for reducing the MS production in potato has been developed, attention should be paid to the reproduction in the other crops in the potato-based rotations too. So many MS are produced in wheat and barley that both the aerial and the subterranean parts can maintain a significant population in the soil. Since the recovery of the MS on the plates was very low, it would be worth investigating the effect of sanitation measures on the soil inoculum density in these crops too.

Due to the large variation between experiments and the existence of some degree of host specificity (Mol *et al.*, 1995), the values obtained are not the maximum potential values for the crops grown. The inoculum density used was not very high, but because root growth in pots is abnormal, the roots in these experiments may have been more susceptible to infection than the roots of plants in the field. The large differences in MS formation among potato cultivars implies that in a rotation, the choice of potato cultivar is an important factor. Other authors have also found large differences between potato cultivars: Slattery (1981), Davis *et al.* (1983) and Mol & Scholte (1995a).

The results presented above cannot be considered to be conclusive for all field situations, but they can serve as a base for modelling work at crop and farm level for different crops, grown under comparable conditions.

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