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Heat treatment to control Colletotrichum acutatum on corms of Anemone coronaria

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Abstract

Anemone corms may be infected with the fungus *Colletotrichum acutatum*, which under certain conditions causes leaf curl and leaf necrosis.

A hot-water treatment (hwt) of infected corms for 1.5 h at 47.5 °C or 1 h at 50 °C suppressed the disease almost completely. The period of application of hwt between harvest and planting time of corms did not influence the efficacy of hwt. Germination potential of different lots of untreated anemone corms varied between approximately 65% and 95%. Hot-water treatment for 1.5 h at 47.5 °C or 1 h at 50 °C reduced germination only slightly if corms of lots that germinated well without hwt were used. However, germination after hwt was severely reduced if lots with a poor germinating potential were used. Germination potential of hwt-treated corms was restored when they were stored for 4 days in moist vermiculite at 20 °C between hwt and planting. Drying of hwt-treated corms after storage in moist vermiculite reduced germination.

A dry heat treatment of infected corms reduced disease incidence too, but results were inconsistent and germination of corms was considerably reduced when control of the fungus was optimal.

Additional keywords: thermotherapy, heat tolerance, corm germination, hot-water treatment.

Introduction

Anemone leaf-curl disease, caused by *Colletotrichum acutatum* (Fig. 1), is characterized by necrosis and downward curling of leaves and by twisting and coiling of petioles and peduncles (Woodcock and Washington, 1979). The disease is spread with contaminated corms (Tramier and Bettachini, 1980). Tramier and Bettachini (1980) supposed that propagules of *C. acutatum* were mainly located in remnants of old leaves around the apex of corms and that the presence of the fungus in the corm under the epidermis was exceptional. Linfield and Price (1983) suggested that the fungus grows systemically through the whole plant. Doornik and Booden (1990) concluded from results obtained after plating corm tissue from various depths that most of the infections with *C. acutatum* were on the outside of the corms. Mycelium had only occasionally penetrated the apical meristem and a few cell layers underneath. This was confirmed by histological studies (A.W. Doornik, unpublished results).

Several researchers reported that this superficial contamination could be reduced by a heat treatment of corms. Woodcock (1978) reported an effective control of *C. acutatum* by hot-water treatment (hwt) for 15 min at 50 °C after soaking the corms for 4 h at 20 °C. Gullino et al. (1981) found a reduction of disease incidence in plants from spore-inoculated corms after hwt for 30 min at 40 or 50 °C. Germination of corms, however, was

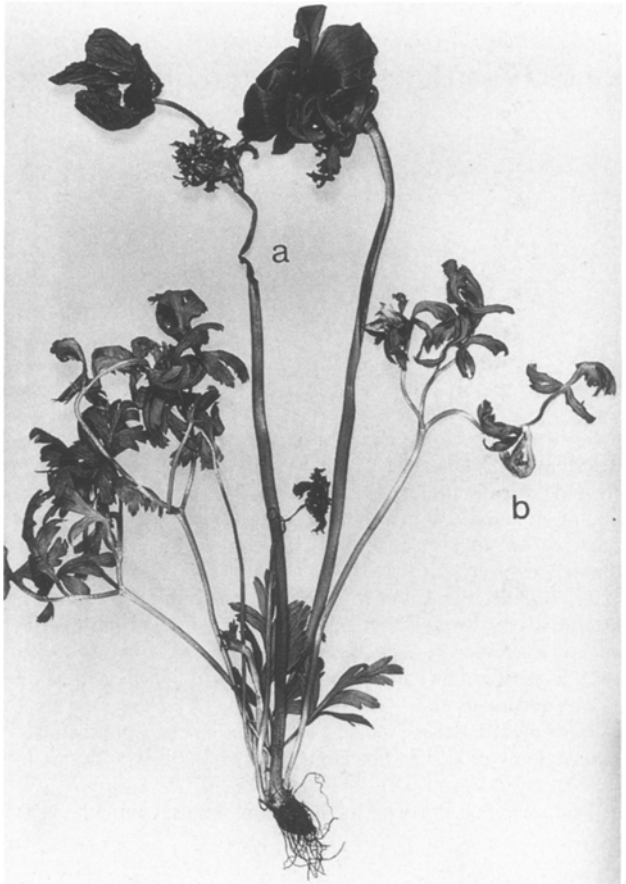

Fig 1. Anemone plant infected by *Colletotrichum acutatum* showing necrosis (a) and curling leaves (b).

reduced too. Vigodsky-Haas et al. (1983) could not isolate any viable *C. acutatum* from infected anemone stems after a hwt of the stems for 15 min at 50 °C. Germination of corms grown in Israel was not affected by a hwt for 15 min at 55 °C applied between July and September. De Winter (1983, 1984) also described a reduction in disease incidence in naturally infected corms after a hwt at 47 or 50 °C. In some trials he found a severe reduction of corm germination after hwt.

A hwt that reduces infection by *C. acutatum* but also severely reduces corm germination is not suitable for practical application. Vreeburg (1978, 1988) found a dry heat treatment at certain temperatures before hwt of daffodil bulbs to be effective against yield reduction caused by hwt.

Pullman et al. (1981) found for several plant pathogens a linear relationship between logarithm of time required to kill 95% of fungal propagules and temperature. When a similar relationship could be found for thermal death of *C. acutatum* as well as for the best germination of corms after hwt, it would be possible to find the most effective treatment for killing the fungus while sparing the host.

In this paper results are presented of a detailed study on the effects of hwt of contaminated corms on the disease incidence and germination of corms.

Materials and methods

Hot-water treatment of spores. The treatments were given to suspensions in a physiological salt solution of spores of 1-week-old cultures. Concentration of the suspensions was 10^6 spores per ml. Three viable isolates of *C. acutatum* were tested. Ten-ml test tubes containing 10 ml of a spore suspension, closed with a lid, were almost completely submerged in a waterbath. Temperatures of the suspension in the tubes and of the water in the waterbath were measured by thermocouples and registered continuously during the experiment on a Kipp flatbed recorder. Time registration of hwt started when the temperature in the suspension was equal to the temperature of the surrounding water. Treatments shorter than 1 min could not be realised accurately and were avoided. The suspensions were subsequently plated out on malt agar (Runia et al., 1988) and incubated at 25 °C under fluorescent light (Philips TLD 36W/33). Colonies were counted after 3 days.

Inoculation with surviving spores. In order to test whether spores of *C. acutatum* after a hwt still could infect healthy corms, samples of the suspension treated in hot water were plated on potato dextrose agar. The suspension was then stored for 3 days at 2 °C until the colonies of the plated samples could be counted. From previous experiments it was known that germination of spores was reduced by a factor 10 after 3 days storage at 2 °C (data not shown). When it was known how many spores had survived the hwt, the stored suspension was concentrated by centrifuging at 1000 rev/min and the sediment resuspended to the required concentration of viable spores, taking into account the effect on germination of storage at 2 °C. Leaves were inoculated with 0.01 ml suspension containing 50 viable spores immediately after emergence when they were still furled. When the suspension had dried, the plants were kept at approximately 100% RH and 25–30 °C during approximately 4 h and then at 18 °C in the greenhouse. Necrose symptoms were recorded after 7 days.

Corms were inoculated on the apex with 0.05 ml suspension containing 500 viable spores and planted immediately. In the experiments with untreated spores, corms were dipped in a spore suspension. By measuring the amount of water absorbed by each corm during 5 min dipping, the spore concentration of the suspension was calculated in order to bring approximately 25 000 spores on each corm. Necrose symptoms were recorded 4–5 weeks after emergence of plants.

Hot-water treatment of corms. To prevent spreading of spores among replicates during hwt, each replicate sample of naturally contaminated corms was put in a plastic bag filled with water of almost the required temperature and placed in a bath with water of the required temperature. The treatment started when water temperature in the plastic bag was the same as in the main bath. Healthy corms were treated in gauze bags in the waterbath.

Storage of corms. Harvested anemone corms were stored at 17 °C and 45% RH. Dry temperature treatments before or after hwt were given in storage rooms where temperature but not RH could strictly be controlled; RH is given in approximate values. Incu-

bation at approximately 90% RH was realized by storing corms in plastic bags together with moist vermiculite (1 l water added to 5 l vermiculite, size 3). For 1000 corms 5–6 l vermiculite was used.

Planting of corms. Except when stated otherwise, corms were planted on the day of hwt. Fifty corms were planted in each of six replicates per treatment. The corms were planted separately in 4 cm pots in order to avoid root contact (Doornik, 1985). In the experiments of which the results are presented in Tables 1 to 3, all planted corms were kept at 9 °C for one week and subsequently placed in the greenhouse at 18 °C . In the course of the experiments it turned out that planting in the greenhouse directly after hwt gave better germination of corms. Consequently, in the experiments reported in Tables 4 and 5, corms were planted and directly placed in the greenhouse. Germination of corms was assessed approximately 4 weeks after planting.

Results

In the field, infected corms may produce leaves with symptoms of necrosis, curled leaves and leaves without these symptoms. Only very rarely corms in the field produce curled leaves without necrotic leaves. In the greenhouse, curled leaves are often found in plants without necrotic leaves, even in plants from untreated, healthy corms. No relationship was found between the occurrence of curled leaves in healthy or infected plants and any of the treatments given. Symptoms of necrosis were only observed in infected plants and are considered to be the more reliable symptoms caused by *C. acutatum* under greenhouse conditions. Therefore, only data are presented on plants showing symptoms of necrosis.

Hot-water treatments of spores in vitro. Ninety-nine percent of the spores were killed after hwt of at the utmost 3 h 42.5 °C, 21 min 45 °C, or 1 min 47.5 °C (Fig. 2). At temperatures from 50 to 60 °C more than 99% of the spores were killed after the shortest treatment given (1 min). Complete kill of the spores was not achieved, not even after 3 min hwt at 60 °C. Hot-water treatment of three isolates, made in 1981 and 1986, gave a similar response. In further trials, only the 1986 isolate was used. Spores of a 7-day-old culture and a 30-day-old culture did not differ in their response. Neither was a difference found for spore concentrations of 10^4/ml or 10^6/ml.

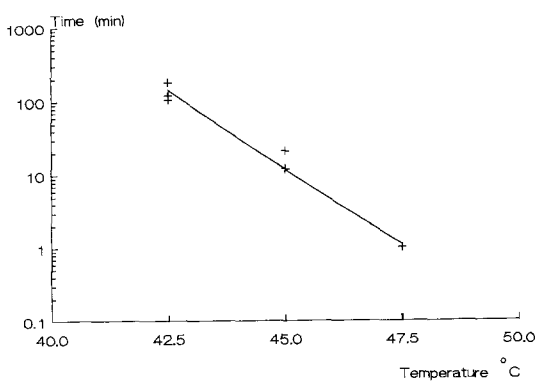

Fig 2. Relation between temperature of hot-water treatment and time required to kill 99% of the spores of *Colletotrichum acutatum*. Data of three replicate treatments are presented. The calculated line is characterized by the equation $\log y = 20.16 - 0.424x$ ($r = 0.998$).

The isolate *C. acutatum*, used for hwt, formed spores from which six differently coloured colonies developed. All six types of colonies developed from spores that survived a hwt that killed more than 99% of the total number of spores.

Spores from three types of colonies were treated separately for 15 min at 52.5 °C. Spores harvested from colonies that had developed from spores that survived this hwt were again submitted to a hwt of 1–15 min at 47.5 or 55 °C. They did not differ in their survival from spores from the original cultures.

Inoculation with surviving spores. Ten percent of the very young furled leaves, inoculated immediately upon emergence, with spores that survived a treatment at 50 °C for 15 min showed necrosis, whereas 40% of the furled leaves showed necrosis after inoculation with the same number of untreated spores.

When healthy corms were inoculated on the apical meristem with 500 spores that survived a treatment at 50 °C for 15 min, no symptoms of necrosis on leaves were found, while 15% of the corms inoculated with the same number of untreated spores formed leaves with symptoms of necrosis.

In a further experiment, both healthy corms and spores were given a hwt of 15 min at 50 °C. When the treated corms were inoculated with 500 surviving spores, again no necrosis was found, whereas 41.9% necrosis was found when hot-water-treated corms were inoculated with 500 untreated spores.

Increase of susceptibility of corms after hot-water treatment. Healthy corms were treated for 30 min at various temperatures and subsequently inoculated by dipping in an untreated spore suspension. The percentages of plants with symptoms of necrosis increased with increasing temperatures of hwt (Table 1). Storage for a week at 20 °C and approximately 40% RH between hwt of healthy corms at various temperatures and inoculation with untreated spores did not decrease susceptibility to infection. Compared with healthy corms that were inoculated immediately after hwt, no difference was found in percentages of plants showing symptoms of necrosis. Results were comparable with those in Table 1.

Hot-water treatment of naturally infected corms. Naturally infected corms had to be treated for at least 1.5 h at 45 °C, or 1 h at 47.5 or 50 °C to reduce the percentage of corms that produced plants with symptoms of necrosis to 1% or less. Hot-water treatments

Table 1. Anemone plants with symptoms of necrosis (%) as influenced by hot-water treatment (hwt)of healthy corms before inoculation with untreated spores of *Colletotrichum acutatum* (approx. 25 000 spores per corm). Six replicates of 50 corms each.

	No hwt	Hwt (30 min)			
		45 °C	47.5 °C	50 °C	52.5 °C
Not inoculated	0 a	–	–	–	–
Inoculated	14.9 b	51.9 c	61.0 d	70.2 e	75.0 e

Data were analysed using regression analysis. Values followed by different letters are significantly different ($P < 0.05$).

Table 2. Anemone plants with symptoms of necrosis (%) as influenced by hot-water treatment (hwt) and month of treatment of corms naturally infected by *Colletotrichum acutatum*. Six replicates of 50 corms each.

Duration of hwt (h)	January				July					
	no hwt	hwt			no hwt	hwt				
		45 °C	47.5 °C	50 °C		45 °C	47.5 °C	50 °C	52.5 °C	55 °C
0	14.1	–	–	–	19.7	–	–	–	–	–
0.5	–	4.8	2.4	0.3	–	5.6	4.8	2.8	0	0
1	–	1.4	0.4	0.7	–	8.3	0.7	0	–	–
1.5	–	1.0	0.3	0	–	0	0	0.4	–	–

Regression analysis revealed only main effects of temperature and exposure time (see text).

applied in January and July gave similar results (Table 2). Main effects of temperature and exposure time were analysed using regression analysis of combined data of both experiments. There was a significant effect of the temperatures and the exposure times of the treatments ($P< 0.05$). The mean percentage necrosis at 45 °C was higher than those at 47.5 and 50 °C. The mean percentage necrosis at 47.5 and 50 °C did not differ. The mean percentage necrosis after a hwt for 0.5 h was only higher than that after a hwt for 1.5 h ($P < 0.05$). There was no difference between a 1.0 h and a 1.5 h hwt. Experiments following those of which the results are shown in Table 2 with six different lots of naturally contaminated corms at various dates have shown that a hwt of 1.5 h at 47.5 °C or 1 h at 50 °C reduced the percentage of plants with necrotic leaves to less than 1%, while the untreated lots produced 10–30% necrotic plants.

Presoaking of corms. Presoaking of corms may improve the results of hwt (Schenk, 1961). However, soaking of the stone-hard, dry anemone corms in water at 20 °C during 30 min to 24 h before hwt did not reduce the percentage of plants with symptoms of necrosis. Moreover, germination of presoaked corms sometimes decreased considerably.

Germination of corms. Influence of hwt on germination of corms showed considerable variation (Table 3). Certain lots germinating for more than 90% when untreated, only showed a slight reduction in emergence after hwt at high temperatures, while other well-germinating lots showed a considerable influence of hwt on emergence. All stocks that germinated poorly without hwt showed a strongly reduced emergence after hwt.

Treatments to reduce adverse effects of hot-water treatment on germination of corms. In the following experiments, corms were treated in hot water for 1.5 h at 50 °C, which strongly reduced germination. The plants from germinated corms did not show necrosis, so no data on necrosis are presented.

A treatment of the corms of 1 week at 30 °C and approximately 30% RH before hwt reduced corm germination significantly more than a hwt without pretreatment (Table 4).

A treatment of 1 week at 9 or 20 °C and approximately 45% RH after hwt did not affect the germination. A treatment of 1 week at 9 or 20 °C and approximately 90% RH after hwt improved corm germination (Table 5).

Table 3. Germination of four lots of anemone corms, naturally infected by *Colletotrichum acutatum* (%), as influenced by hot-water treatment (hwt). Six replicates of 50 corms each.

Duration of hwt (h)	Well germinating lots						Poorly germinating lots					
	no hwt	hwt					no hwt	hwt				
		45 °C	47.5 °C	50 °C	52.5 °C	55 °C		45 °C	47.5 °C	50 °C	52.5 °C	55 °C
Lot A							*Lot C*					
0	93.0	–	–	–	–	–	73.3	–	–	–	–	–
0.5	–	95.0	98.3	94.7	90.7	82.3	–	73.3	62.7	68.7	54.0	28.7
1	–	93.3	92.6	90.9	–	–	–	63.0	69.7	50.0	–	–
1.5	–	94.3	94.0	82.6	–	–	–	45.3	57.7	26.3	–	–
Lot B							*Lot D*					
0	93.3	–	–	–	–	–	65.0	–	–	–	–	–
0.5	–	–	87.3	93.0	57.0	–	–	–	48.3	13.3	2.0	–
1	–	–	76.0	76.3	–	–	–	–	22.7	3.7	–	–
1.5	–	–	72.0	23.0	–	–	–	–	11.0	0	–	–

Data of lot A and C (1988) were analysed together using analysis of variance as were the data of lot B and D (1989). There was a significant interaction between treatments and germination of the four lots ($P = 0.001$). Germination of lot A and B was significantly better than that of lot C and D. Least significant difference at $P = 0.05$ of lot A and C was 8.5, of lot B and D 8.9.

Table 4. Germination of anemone corms (%) as influenced by storage for 1 week at 30 °C or at 9 and 20 °C before or after hot-water treatment (hwt, 1.5 h at 50 °C). Six replicates of 50 corms each (germination potential of the lots of anemone corms varied between 80 and 90%).

Pretreatment	Treatment after hwt (temp./RH)			
	None	9 °C/45%	20 °C/45%	Mean
None	40.3	44.3	39.0	41.2
30 °C, 30% RH	32.7	28.6	30.7	30.7

Data were analysed using analysis of variance. There was a significant main effect of pretreatment ($P < 0.001$). Least significant difference of means was 5.3.

Table 5. Germination of anemone corms (%) as influenced by storage for 1 week at two temperatures and relative humidities after hot-water treatment (hwt, 1.5 h at 50 °C). Six replicates of 50 corms each (germination potential of the lots of anemone corms varied between 80 and 90%).

No storage	Storage conditions (temp./RH)			
	9 °C/45%	20 °C/45%	9 °C/90%	20 °C/90%
40.3 a	44.3 a	39.0 a	67.7 b	76.7 b

Data were analysed using analysis of variance. Values followed by different letters are significantly different. Least significant difference at 5% level between treatments was 9.2.

Table 6. Germination of anemone corms (%) as influenced by various periods at 90% RH and different temperatures after hot-water treatment (hwt) at 50 °C during 1.5 h. Six replicates of 50 corms each.

	Temperature after hwt (°C)	Length of period (days) at 90% RH after hwt				
		0	2	4	6	9
control (no hwt)	_	78.7 f	_	_	_	
hwt[a]	_	11.7 a	_	_	_	_
hwt	2	_	5.7 a	_	41.0 c	38.7 c
hwt	9	_	27.0 b	65.3 d	67.0 de	_
hwt	20	_	58.0 d	78.0 f	75.0 ef	_

[a] Planted immediately after hwt.
Values followed by different letters are significantly different. Least significant difference at 5% level = 9.6

Bacterial rot was sometimes noticed on some of the corms after 1 week at 20 °C and approximately 90% RH. In a following experiment corms were stored after hwt at three different temperatures and approximately 90% RH during various periods. For all periods storage at 20 °C gave the best results (Table 6). As bacterial rot was less after 4 days storage than after 6 days and germination was better after 4 days than after 2 days storage, 4 days storage at 20 °C and approximately 90% RH was chosen as a standard treatment (st) after hwt.

Drying after hot-water treatment and standard treatment (4 days at 20 °C and 90% RH). When hwt is given in practice during winter, corms have to be stored till planting. However, after hwt and st many corms showed a beginning outgrowth of roots and swelling of the apical meristem. During storage corms gradually dry out. In experiments it was shown that drying during storage of corms after hwt and st tended to reduce germination after planting.

Dry-heat treatment. As another method to control *C. acutatum* in *Anemone* a dry-heat treatment was tried out. Dry-heat treatments of hyacinth bulbs in storage rooms at temperatures between 30 and 44 °C reduce infection of *Xanthomonas hyacinthi* in hyacinths (Vreeburg and Kamerman, 1980). Dry-heat treatment of infected anemone corms at 30 or 40 °C reduced the percentage of plants with symptoms of necrosis, but results of replicate experiments were inconsistent. Germination of heat-treated corms was considerably reduced when control of *C. acutatum* was optimal (data not shown).

Discussion

The logarithmic relation between time and temperature of heat treatments and the thermal death of some plant pathogens, reported by Pullman et al. (1981), was found also for the thermal death of spores of *C. acutatum* (Fig. 2).

A heat mortality curve of spores in vitro may give indications at which temperature and duration a hwt of infected plant material could be successful. However, spores of *C. acu-*

tatum in suspension were killed at much lower temperatures and shorter periods than propagules of the fungus in or on anemone corms (Fig. 2 and Table 2). This large difference between the effects of hwt of spores in vitro and on corms may be explained by the fact that the tissues infested with spores or mycelium are often well protected by remnants of old leaves around the apical meristem. Complete elimination of spores in suspension was rarely achieved, even by hwt for 3 min at 60 °C. It is difficult to explain this phenomenon. Selection of genetically thermoresistant spores does not account for it, because a hwt of offspring of spores surviving hwt and a hwt of the original spores gave the same results.

Under conditions favourable to infection, inoculation of very young furled leaves, with spores that survived a hwt of 15 min at 50 °C occasionally resulted in necrosis. Inoculation of healthy corms with surviving spores did not result in necrosis. Inoculation of healthy corms after hwt with spores that survived hwt was not successful either. These results are in contrast with those obtained with spores of *C. acutatum* that did not receive hwt (Table 1) and indicate that spores surviving hwt are less pathogenic and not a source of corm infection. To draw firmer conclusions, more data are required on the effect of a 3-day storage at 2 °C on the capacity of these surviving spores to infect leaves or corms (see 'Materials and methods').

Some, but not all, lots of corms showed a decrease in germination after hwt, depending on temperature and duration of hwt. This decrease was especially pronounced in lots of corms with low natural germination potential. However, the relation between germination potential of untreated corms and the effect of hwt on germination was not completely predictable. Vreeburg (1978, 1988) found that temperature treatments before hwt of daffodils reduced losses caused by hwt. Anemone reacted adversely on such pretreatments. A treatment at approximately 90% RH after hwt of anemone corms showed an increased germination with increasing temperature and time (Tables 4 and 5). This phenomenon may be interpreted as a curing effect of a high-RH treatment on hwt-damaged meristems. This response is in accordance with that reported by Roebroeck and De Greeff (1987) and Roebroeck et al. (1987), who observed that lily scales kept under moist conditions at 17 °C or higher, immediately after they had been broken from bulbs, were not infected by *Penicillium hirsutum*. This was due to wound-healing, which was not achieved under dry conditions.

The corms planted immediately after hwt in soil kept moist by daily watering germinated less than corms that were stored for 4 days in moist vermiculite. Relative humidity around corms under both experimental conditions will have been high. The difference in germination may be due to presence of bacteria or other contaminants in soil, whereas vermiculite is almost sterile. It is speculated that contaminants in soil may have penetrated the damaged apical meristem of the corms before wound-healing could have taken place.

After 4 days at 20 °C and approximately 90% RH, swelling of the apical meristem and outgrowth of roots indicated that the growing process of anemone corms was initiated. Not surprisingly, drying of corms after hwt and st tended to reduce germination. Hot-water treatment and st may be effectuated with good control of *C. acutatum* at any date preferred by the grower. Reduction of germination by drying after st limits the choice of treatment date. By storing the corms in moist vermiculite at 2 °C after st, the planting date can be postponed by approximately 4 weeks without an adverse effect (Doornik et al., 1990).

All trials reported here were performed under greenhouse conditions. Field experiments

confirmed greenhouse results. In these field trials, the production of flowers and of seed were also recorded as parameters for plant growth (Doornik et al., 1990). Flower production was not reduced by hwt and st as compared with flower production by control corms. In these field experiments more flowers and seed tended to be formed when corms were treated in hot water at the end of April than when treated at the beginning of April.

From the results of the experiments it is concluded that hwt of anemone corms for 1.5 h at 47.5 °C or 1 h at 50 °C followed by 4 days at 20 °C in moist vermiculite is an effective method to control *C. acutatum*.

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