Screening adjuvants for use in spray solutions of entomopathogenic nematodes through different parameters: EPN survival, infectivity, sedimentation speed and leaf deposition

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SUMMARY

Due to more stringent regulations on pesticides, biological alternatives for pest control are gaining popularity. Entomopathogenic nematodes (EPN) are a promising biological alternative to conventional insecticides. However, EPN are not always equally effective due to environmental factors like temperature, humidity and sunlight. The inclusion of adjuvants in the spray liquid has proven to enhance the effectiveness of EPN in various crops. This paper proposes a selection procedure for spray adjuvants that are to be used for outdoor applications of entomopathogenic nematodes. The selection procedure tests the effect of adjuvants on the survival of EPN, their infectivity and their sedimentation speed. The results of the tests for several types of surfactants, humectants and UV-protective adjuvants are presented in this paper. The two best scoring surfactants and the two best scoring humectants were selected for further testing. Subsequently, the effect of the four selected adjuvants (separate and in surfactant-humectant combination) on the deposition of EPN on cabbage and leek leaf discs was tested.

Key words: entomopathogenic nematodes; adjuvants; survival; deposition

INTRODUCTION

As a result of the more restrictive regulations on pesticides, there are new opportunities for biological alternatives regarding pest control methods. Entomopathogenic nematodes (EPN) are a promising biological alternative to conventional insecticides, although not always equally effective, due to the adverse effect of environmental factors like temperature, humidity and sunlight. The use of adjuvants when applying EPN has proved to enhance the effectiveness of EPN against pest insects in various crops. In this paper we propose a selection procedure for adjuvants to be used for outdoor applications of entomopathogenic nematodes.

MATERIALS AND METHODS

The first test is a measurement of the survival percentage of two species of entomopathogenic nematodes, *Steinernema feltiae* and *Steinernema carpocapsae*, suspended in a spray solution. The second test is an infectivity test of the abovementioned nematodes. In this test, nematodes suspended in spray solution are put

together with larvae of *Galleria mellonella* in a one-on-one ratio. The mortality of the larvae is measured 7 days after of incubation. The third test measures the sedimentation speed of *S. feltiae* in spray solutions containing adjuvants influencing viscosity. The fourth test measures the influence of adjuvants on the deposition of nematodes on leaves.

Adjuvant preselection

A set of 17 adjuvants with different attributes was selected for testing (Table 1).

TABLE 1: Selected adjuvants

Name	Chemical composition	Function
Synperonic 91/5	C9-11 alcohol-(5)-ethoxylate	Spreading/wetting
Synperonic 91/6	C9-11 alcohol-(5)-ethoxylate	Spreading/wetting
Synperonic 10/6	C10 alcohol-(5)-ethoxylate	Spreading/wetting
Atplus 245	C9/C11 alcohol ethoxylaat/propoxylate	Spreading/wetting
AL-2575	C8/C10 polysaccharide	Spreading/wetting & humectant
Crodasinic LS-30	Sodium lauroyl sarcosinate	Spreading/wetting
Adinol OT-72	Sodium N-methyl oleoyl taurate	Spreading/wetting
Trend 90	Isodecyl alcohol ethoxylate	Spreading/wetting
Tween 20	Polyoxyethylene-(20)-monolaurate	Humectant
Pricerine 9081	Glycerine	Humectant
PVA	Polyvinylalcohol (PVA) 2000 g/mol	Humectant
TAM-1892	Terpene polymer	Humectant/sticker
Xanthan	Xanthan gum	Humectant
Synperonic PE/F108	EO/PO block copolymer	Dispersant
Synperonic PE/F127	EO/PO block copolymer	Dispersant
Clearshield UV 390B	Polymer (no further details available)	UV blocker
Solaveil Clarus 30W	Titaniumdioxide	UV-A blocker

Nematode survival

To measure the survival of the nematodes in the spray solution, the following protocol was used. A quarter of a unit of nematodes (1 unit = 50 mio nematodes) was dissolved in 5 L of water resulting in a concentration of 2.5 million EPN/L. To avoid settling of the nematodes the water was kept turbulent by bubbling air through the solution. After 30 minutes, the solution was stirred thoroughly and two 200 mL samples were taken and transferred to 250 mL round-bottom flasks. An amount of 0.2 g of the adjuvant under investigation was added to one of the flasks. Both flasks were put on a shaker at 120 rpm. After 2.5 hours, a subsample of 100 µl of each flask was taken and transferred to a separate counting plate. Tap water was added to the plates until the bottom of the plates was completely covered. The nematodes were left for a moment to settle onto the plate. Subsequently all nematodes on the plate were counted. Afterwards, the plate was recounted, but this time only the dead nematodes were counted. Nematodes were considered dead if they did not show movement after being prodded three times. This procedure was repeated three times. The absolute survival percentages of all counted plates were calculated and recorded and mean survival percentage on the plates containing the nematode-adjuvant solution was compared to that of the plate with no adjuvant. The test was performed for two EPN species: *S. feltiae* and *S. carpocapsae*. This test was repeated with one adjustment: the time on the shaker was set at 15 hours (worst case scenario).

Nematode infectivity

To measure nematode infectivity in the spray solution, the following protocol was used. For each adjuvant, a set of 3 multi-well plates was filled with 2 g of sand per well. An additional set of 3 plates was prepared for the reference treatment with water. A quarter of a unit of nematodes was dissolved in 5 L of water. To keep the nematodes from settling, the water was kept turbulent by bubbling air through the solution. After 5 min the solution was stirred thoroughly and 200 mL samples were taken and transferred to 250 mL round-bottom flasks. to which 0.2g of a specific adjuvant had already been added. The flasks were put on a shaker at 120 rpm for 30 min. A subsample of 100 µL of each flask was taken and transferred to a separate counting plate and water with adjuvant was added until the bottom of the plates was completely covered. From this solution, one nematode in 120 µL of spray solution was transferred to a sand-filled well. This was repeated for all the wells in the set of plates designated to a specific adjuvant. *Galleria mellonella* larvae were added to the plates, one larva per well. Afterwards, the plates were closed with a lid and parafilm. The plates were incubated at 24°C for 7 days after which the number of infected larvae per plate could easily be determined due to the color of the larvae. *Galleria*-larvae, infected with *Steinernema* spp. tend to be brownish black in color; larvae infected with *Heterorhabditis* larvae tend to be red. Infection percentages per plate were recorded and the mean infection percentage per adjuvant could be determined and compared to the other treatments.

Nematode sedimentation speed

To measure the sedimentation speed of nematodes in the spray solution, the following protocol was used. A quarter of a unit of nematodes was dissolved in 5 L of water. To keep the nematodes from settling, the water was kept turbulent by bubbling air through

the solution. After 30 minutes, the solution was stirred thoroughly and five 200 mL samples were taken and transferred to five graduated 250 mL cylinders. The cylinders were closed and turned ten times to ensure a uniform dispersion of nematodes. The cylinders were left to rest for 0, 1, 2, 5, 10 and 20 min respectively. Afterwards, the upper 200 mL of spray solution was drained from the cylinders, leaving only the lower 50 mL in the cylinders. The upper 200 mL of each cylinder was transferred to a 250 mL round-bottom flask and put on a shaker at 120 rpm. A subsample of 100 µL of each flask was taken and transferred to a separate counting plate. Tap water was added to these plates until the bottoms of these plates were completely covered. The nematodes were left for a moment to settle onto the plate. Subsequently all nematodes on the plate were counted and the concentration in the upper 200 mL of each cylinder was calculated. If the number of nematodes on the plate was lower than 200, a bigger sample was taken and counted. Both *S.feltiae* and *S. carpocapsae* were tested for sedimentation speed. *S. feltiae*, the biggest nematode species, sedimented faster than *S. carpocapsae* and this is why all tests with adjuvant added to the spray solution were carried out with *S. feltiae*. All adjuvants listed as humectants and dispersants were included in the test. Adjuvants were added at a concentration of 0.3 g/L. The test was repeated at 0.5 g/L adjuvant for the most successful sedimentation retarding adjuvants.

Nematode deposition on leaf discs

To measure the deposition of nematodes on leaves, the following protocol was used. Leaf discs of leek and two types of cabbage (*Brassica oleracea, Botrytis* cultivar group and *Brassica oleracea convar. capitata var. sabauda*) with a diameter of three cm were cut out of freshly cut plant leaves. The leaf discs were fixed in clamps at a certain angle to vertical. The leaf discs were sprayed with a nematode solution (2500 nematodes/mL) at a dose of 1095 L/ha.

Six different adjuvant solutions were tested, plus one reference solution without nematodes. The six treatments were:

- A solution of *S. feltiae* with Crodasinic LS-30 (1 g/L) on leek
- A solution of *S. feltiae* with Tween 20 (0,3 g/L) on leek
- A solution of *S. feltiae* with Crodasinic LS-30 (1 g/L) and Tween 20 (0,3 g/L) on leek
- A solution of *S. carpocapsae* with Adinol OT-72 (1 g/L) on the two types of cabbage
- A solution of *S. carpocapsae* with Pricerine 9081 (0,3 g/L) on the two types of cabbage
- A solution of *S. carpocapsae* with Adinol OT-72 (1 g/L) and Pricerine 9081 (0,3 g/L) on the two types of cabbage.

Fifteen inclined leaf discs were examined per plant species and treatment for coverage and survival. The leek discs were placed at a 15° angle to vertical, and covered on the lower side with filter paper. The discs of the cabbage plants were put at a 45° angle to vertical, and were covered on the upper side with filter paper. The reason for the different inclination is the normal leaf configuration of leek (close to vertical) and cabbage (ranging between horizontal and vertical). The reason for covering with filter paper was to only measure deposition on one side of the leaves. In leek, the upper side of the leaves was aimed for to simulate control of thrips. In cabbage, the lower side of

the leaves was aimed for, to simulate control of caterpillars, which can usually be found foraging on the lower side of the leaves.
Immediately after spraying, three discs were collected per plant species placed in a Petri dish filled with water with the exposed side downward. The other discs were placed with the sprayed side up in empty Petri dishes and incubated at 24°C and 60% humidity. After 1, 2, 3 and 4 h, three discs per plant species were collected from the incubator and transferred to Petri dishes filled with water with the sprayed leaf side downward. Afterwards, the leaf discs were removed from the Petri dishes, and all nematodes present in the Petri dishes were counted. Deposition was calculated and compared to the deposition of the reference treatment. All experiments were repeated three times.

RESULTS AND DISCUSSION

Nematode survival

Figures 1 & 2 show the results of the survival tests of *S. carpocapsae* after three and 15 h in the spray solution and Figures 3 & 4 show the corresponding results for *S.feltiae*. Generally, *S. carpocapsae* was less sensitive to adjuvants. Remarkable results were obtained for the alcohol ethoxylate adjuvants and for the polysaccharide adjuvant, AL-2575. After 3 h, 3 out of 4 alcohol ethoxylate adjuvants showed a large decrease (>50%) in mobility. Nematodes in the AL-2575 solution showed no mobility at all. These nematodes were however, not dead, because after 15 hours, mobility of the nematodes was back at the level of the reference treatment. The best overall spreader adjuvant for *S. carpocapsae* after 3 h was Adinol OT-72. After 15 h, the mobility of *S.carpocapsae* dissolved in this adjuvant remained at the mobility level of the reference sample.
A different picture revealed for *S. feltiae*. Atplus 245, an alcohol ethoxylate, seemed to be the best adjuvant after 3 h, however after 15 h, no mobility was observed. A possible explanation for the high initial mobility (>140%) might be an irritating effect of Atplus 245 on *S. feltiae*. All other alcohol ethoxylates showed a large decrease in mobility both after 3 and 15 h. The nematodes that were mixed with Crodasinic LS-30 showed a good survival. Their survival remained at 90% of the survival of the reference sample after 15 hours. This adjuvant seems to be the best option for enhancing the spreading of *S. feltiae* spray solutions over leaves.
Humectant and dispersant adjuvants showed little effect on the mobility of any of the nematode species. One exception, however, is the terpene polymer adjuvant, TAM-1892, which showed a very strong immobilising effect on *S. feltiae* nematodes. This adjuvant was left out of the sedimentation tests due to these results.

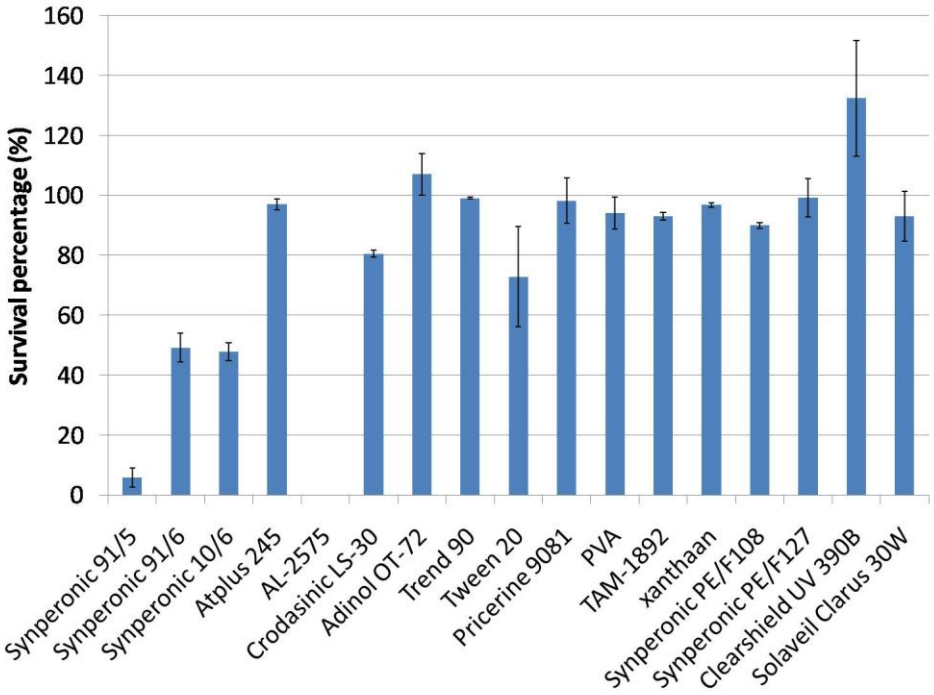

Figure 1: Relative survival percentages (±SE) of *S. carpocapsae* after a three hours stay in spray solution.

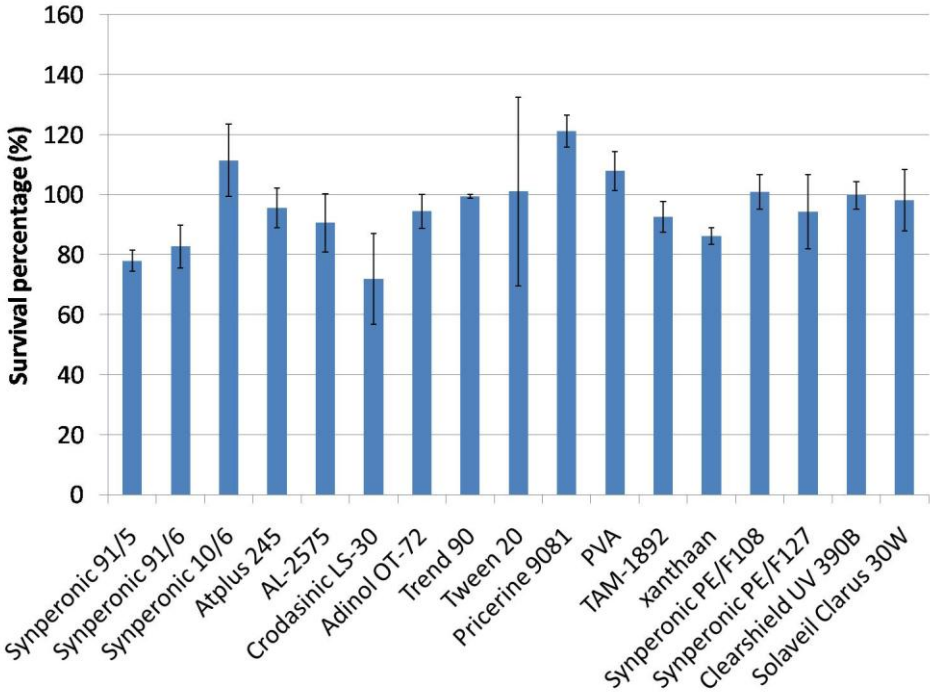

Figure 2: Relative survival percentages (±SD) of *S. carpocapsae* after a 15 hours stay in spray solution.

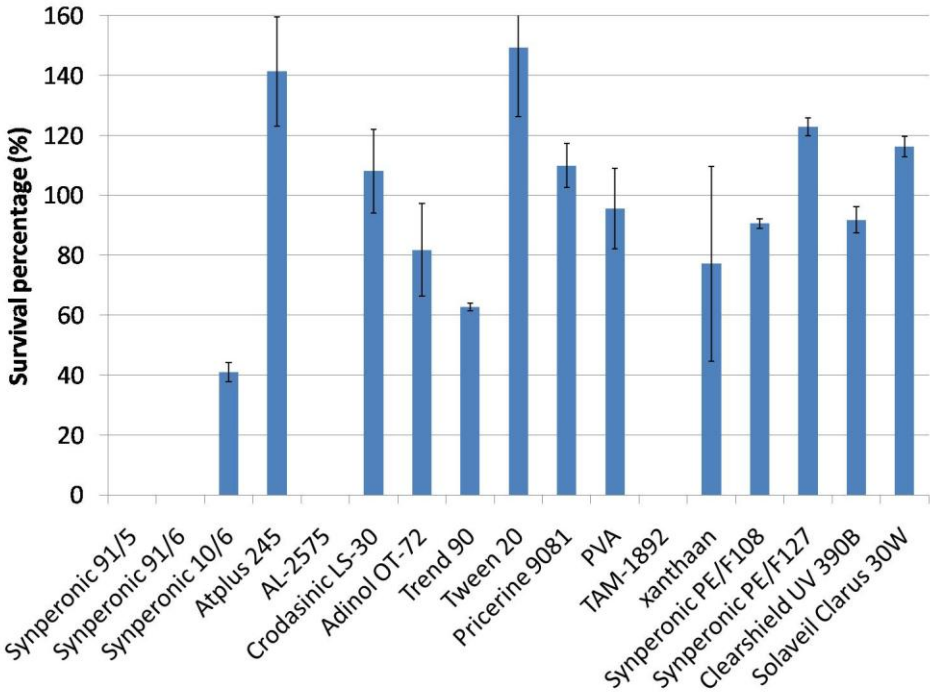

Figure 3: Relative survival percentages (±SD) of *S. feltiae* after a three hours stay in spray solution.

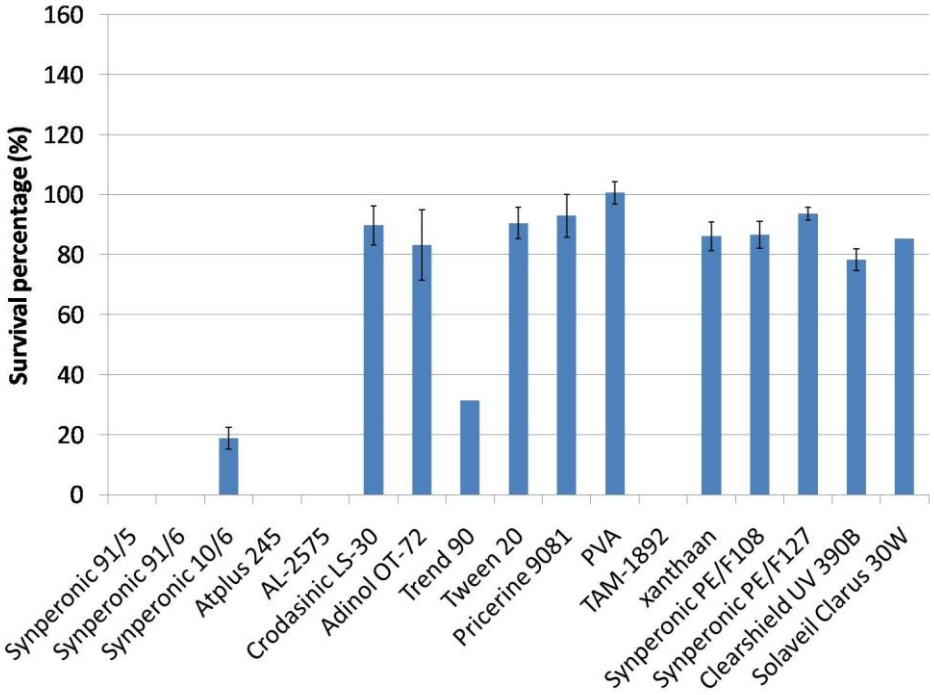

Figure 4: Relative survival percentages (±SD) of *S. feltiae* after a fifteen hours stay in spray solution.

Nematode infectivity

In the one-on-one nematode infectivity test, the infectivity in the control as well as the adjuvant treatments was less than 30%. High variability between test plates was recorded and consequently no significant differences between treatments were found. It was concluded that this test was unsuitable for adjuvant selection.

Nematode sedimentation speed

An exponentially decreasing concentration curve with a very high correlation coefficient (R^2=0.96) was found for the non-adjuvant treatment and from the exponential curve a concentration half-time could be calculated. The same relationship was found for the adjuvant treatments. The resulting half-times are shown in Table 2.

TABLE 2: Half-time values for spray solutions of *S. feltiae* with selected adjuvants.

Adjuvant	Half-time (s)
H_20	212,2
AL-2575	226,0
Tween 20 (0,3 g/l)	315,1
Tween 20 (0,5 g/l)	284,9
Pricerine 9081	268,3
PVA 2000 g/mol (0,3 g/l)	295,0
PVA 2000 g/mol (0,5 g/l)	317,5
Xanthan	3465,7
Synperonic PE/F 108	240,4
Synperonic PE/F 127	217,7

All but one adjuvant had similar half-times around 200-300 s, which is a (slight) improvement of the half-time obtained with the reference solution. Only xanthan gum had a remarkably higher half-time. However, the use of xanthan gum as an adjuvant is problematic because xanthan tends to form clumps, which might clog the spraying equipment. If this problem can be solved xanthan gum should be included in future research.

Nematode deposition on leaf discs

Figure 5 shows that all sprays with adjuvants resulted in somewhat higher plant coverage on leek. Especially inclusion of Crodasinic LS-30 resulted in higher deposits on leek leaves.

The effect of adjuvants on leaf deposits on *Brassica oleracea*, *Botrytis* cultivar group was quite different (Figure 6). Adjuvants reduced plant coverage compared to the reference treatment. On leaves of *Brassica oleracea convar. capitata var. sabauda*, only Adinol OT-72 increased plant coverage (Figure 7).

The nematode survival test ruled out alcohol ethoxylates and a terpene polymer as possible adjuvants for use in EPN spraying. Adjuvants can influence the deposition of nematodes on leaves, positively as well as negatively, and the effects are dependent on

the adjuvant but also on the plant species in question. In the near future, semi-field tests will be conducted to examine if adjuvants can have positive effects on the plant protective properties of EPN.

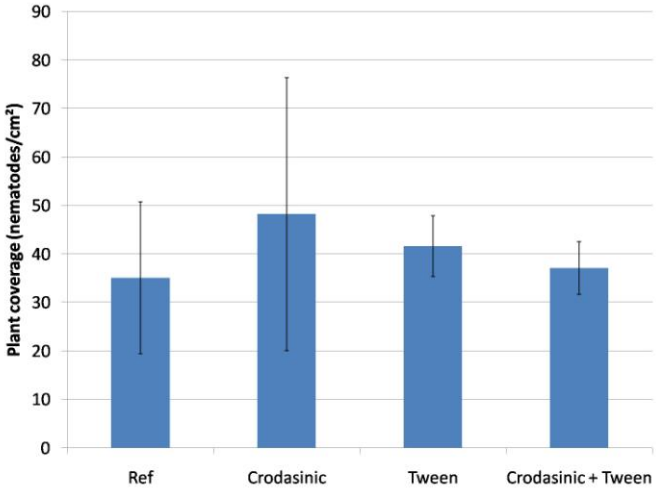

Figure 5: Effects of different adjuvants on plant coverage (±SD) of leek by nematodes of *S. feltiae*.

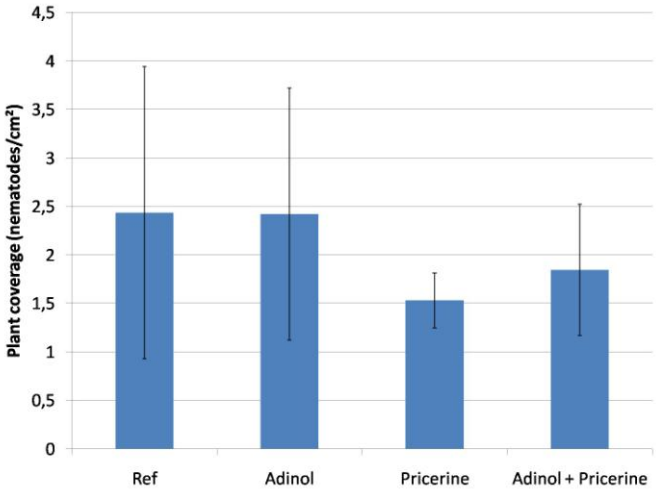

Figure 6 and: Effects of different adjuvants on plant coverage (±SD) of cauliflower (*Brassica oleracea, Botrytis* cultivar group)) by nematodes of *S. carpocapsae*.

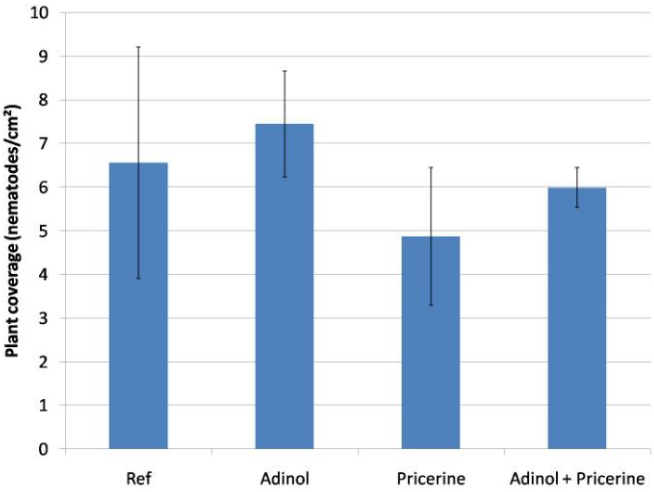

Figure 7: Effects of different adjuvants on plant coverage (±SD) of savoy cabbage (*Brassica oleracea convar. capitata var. sabauda*) by nematodes of *S. carpocapsae*.

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