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Abstract: Chrysanthemum Verticillium wilt caused by V. dahliae is one of the most damaging soil-borne diseases affecting chrysanthemum production in Italy and in Europe. After the definitive phase-out of methyl bromide and the non-inclusion or limitation of other fumigants a great deal of research to identify alternative treatments for controlling soil-borne pathogens has been conducted. One of the possible alternatives is the use of the new fumigant dimethyl disulfide (DMDS), which is also found in minor amounts in nature as a compound that plays a role in the global sulfur cycle. Two greenhouse trials were carried out over two years (2013-2014), to evaluate the efficacy of DMDS (600 kg ha-1) on Verticillium wilt development and chrysanthemum stems yield in naturally infected soil. Treatment with DMDS significantly reduced the incidence of Verticillium wilt compared to the untreated control and results were similar to those obtained with a standard chemical fumigation (Chloropicrin or Metam sodium). Furthermore, stem yields obtained with DMDS and standard chemical fumigations were significantly higher than untreated control. This study shows that V. dahliae was not only isolated from diseased tissues, but also from symptomless tissues in residual chrysanthemum stems. Results suggest the importance of removing chrysanthemum residues infected with V. dahliae, for managing Verticillium wilt and the effectiveness of DMDS fumigation as well as standard chemical fumigations in decreasing V. dahliae residual stems colonization.

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Dr. S.N. Wegulo
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COVER LETTER FOR RESUBMISSION OF MANUSCRIPT CROPRO-D-16-00660

Dear Dr. Wegulo,

enclosed please find our manuscript entitled "**Efficacy of dimethyl disulfide (DMDS) for the control of chrysanthemum Verticillium wilt in Italy**" CROPRO-D-16-00660 revised according to both reviewers' comments/suggestions which we would like to resubmit for publication as **Short Communication** in **Crop Protection**.

The manuscript is being resubmitted by me, Susanna Pecchia (Corresponding author), on behalf of all the authors.
With the submission of this manuscript I would like to undertake that:

- All authors of this research paper have directly participated in the planning, execution, or analysis of this study;
- All authors of this paper have read and approved the final version resubmitted;
- The contents of this manuscript have not been copyrighted or published previously;
- The contents of this manuscript are not now under consideration for publication elsewhere;
- The contents of this manuscript will not be copyrighted, submitted, or published elsewhere, while acceptance by the Journal is under consideration;
- My Institute's (University of Pisa, Department of Agriculture, Food and Environment) representative is fully aware of this submission.
- The iThenticate plagiarism check showed 34% similarity of text in the manuscript to text in published literature. We revised the manuscript, re-writing only the sentences that showed a similarity between 2 and 4%. The sentences with a similarity of 1% are very common in many papers even those that do not fit our subject or we do not cited or consulted (e.g. 15-17, 20-24, 26-50).

We shall look forward to hearing from you at your earliest convenience.

Yours sincerely

Date: 9.11.2016

Susanna Pecchia
Susanna Pecchia

RESPONSE TO REVIEWERS

Reviewer 1

I have only minor comments, all regarding to the figures. The figures should be independent.

Fig. 1. - In the legend give CP its full name.
DONE. WE DESCRIBE IN FULL ALL TREATMENTS IN THE LEGEND.

Fig. 2. Harvested stems (per plant, per plot ?).
DONE.

Fig. 3. - I guess the discovery of the pathogen in 2013 in the chemical treatments was zero. It can integrated as no result. I suggest to add in the legend to the figure something like " no pathogen was recorded in the DMDS and the CP treatment in the 2013 experiment" . only suggestion.
DONE. WE ADD A SIMILAR SENTENCE IN THE LEGEND.

Reviewer 2

My most significant question regards the structure of the experiments. Only two greenhouse trials were carried out in two different years but with two formulations and two different mode of application (shank and drip application). The results appear clear but the experiments were not repeated. I suggest to add in the text that the main activity of DMDS is for the control of nematodes (300-400 kg/ha) and the high dose (600-800 kg/ha) might be effective against fungal pathogens.

COMMENTS ARE GIVEN IN THE TEXT. THE DOSE RATE FOR FUNGI IS 600 KG/HA USING VIF/TIF FILMS. THE DOSE RATE 800 KG/HA WAS USED IN THE PAST WITH NO PLASTIC COVER OR PE FILMS.

In addition, in my opinion the authors emphasize too that DMDS is a natural compound. It should be remembered that the evaluation is always based on the concept that there is an acceptable minimum. So, I suggest to revise the text in the abstract, introduction and discussion sections. Actually, in order to limit the use of fumigants, the new EU Directive on Sustainable Use of Pesticides in agriculture (Directive 2009/128/EC) requires the adoption of integrated pest management, reduction of the dosages and the use of other control strategies to reduce environmental impact. I think that some considerations should be included in the discussion.

MODIFICATIONS DONE IN THE TEXT AS SUGGESTED BY THE REVIEWER

Other changes or further information are requested before the acceptance and are listed below:

Pag. 2 Line 1: please, you change Vericillium with Verticillium
DONE

Pag. 2 Line 11: please, to add comma after Nelson
DONE

Pag. 2 Line 14: please, to check MIPAAF, 2007 or 2009 how is reported in the reference section?
DONE.

Pag. 2 Line 18: to add comma after Rataj-Guranowska and after Ispahani et al.
DONE

Pag. 4 Line 11: How many plants were affected prior to the beginning of the experiments?

IN THE FARM WHERE THE TRIALS WERE CONDUCTED DISEASE INCIDENCE WAS ABOUT 20-30% BUT THIS IS ONLY A ROUGH ESTIMATE BASED ON INFORMATION PROVIDED BY THE GROWER. FOR THIS REASON WE DO NOT INSERT ANY DATA.

Pag. 5 Lines 6-8: please, to add the plot size (row numbers, length and width)
DONE

Efficacy of dimethyl disulfide (DMDS) for the control of chrysanthemum Verticillium wilt in Italy

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ABSTRACT

Chrysanthemum Verticillium wilt caused by *V. dahliae* is one of the most damaging soil-borne diseases affecting chrysanthemum production in Italy and in Europe. After the definitive phase-out of methyl bromide and the non-inclusion or limitation of other fumigants a great deal of research to identify alternative treatments for controlling soil-borne pathogens has been conducted. One of the possible alternatives is the use of the new fumigant dimethyl disulfide (DMDS), which is also found in minor amounts in nature as a compound that plays a role in the global sulfur cycle. Two greenhouse trials were carried out over two years (2013-2014), to evaluate the efficacy of DMDS (600 kg ha^{-1}) on Verticillium wilt development and chrysanthemum stems yield in naturally infected soil. Treatment with DMDS significantly reduced the incidence of Verticillium wilt compared to the untreated control and results were similar to those obtained with a standard chemical fumigation (Chloropicrin or Metam sodium). Furthermore, stem yields obtained with DMDS and standard chemical fumigations were significantly higher than untreated control. This study shows that *V. dahliae* was not only isolated from diseased tissues, but also from symptomless

tissues in residual chrysanthemum stems. Results suggest the importance of removing chrysanthemum residues infected with *V. dahliae*, for managing Verticillium wilt and the effectiveness of DMDS fumigation as well as standard chemical fumigations in decreasing *V. dahliae* residual stems colonization.

Key words: dimethyl disulfide, DMDS, fumigation, *Verticillium dahliae*, soil-borne disease, chrysanthemum

1. Introduction

The genus *Chrysanthemum* belongs to the Asteraceae family and includes more than 30 species, many of which are wild flowers native to Asia and north-eastern Europe (Horst and Nelson, 1997). It is one of the most important flower crops commercially grown as an annual flowering plant throughout the world typically used as cut flower or potted plant.

Italy is the main European producer with a growing area of 1180 ha and a production of 437 million pieces (Chrysanthemum multiflora and bloom) (MIPAAF, 2009). In Tuscany the major areas where it is grown commercially are Lucca and Pistoia provinces (Belletti *et al*., 2008).

Verticillium dahliae Kleb. is one of the most damaging fungal pathogens of chrysanthemum in most chrysanthemum-growing countries (Elena and Pamplomatas, 1998; Korolev *et al*., 2000; Hiemstra and Rataj-Guranowska, 2003; Han *et al*., 2007; Ispahani *et al*., 2008). It is a soil-borne pathogen that causes vascular wilt in over 400 plant species (Agrios, 2004; Inderbitzin and Subbarao, 2014), mainly in the subtropical and temperate climates.

The fungus produces microsclerotia in the dying tissues of the infected plant. These resting structures, agglomerates of thick-walled melanized cells, can survive in the soil for up to 13 years in the absence of susceptible hosts (Schnathorst, 1981; Pegg and Brady, 2002).

Verticillium wilt is traditionally controlled by soil fumigation in order to reduce the density of viable microsclerotia levels below the threshold causing acceptable crop losses (Butterfield *et al*., 1978; Klosterman *et al*., 2009; Wei *et al*., 2015). Anyway in some crops, such as strawberry, even as few as 1 CFU g^{-1} of soil can lead to significant wilt symptoms (Harris and Yang, 1996).

In the past 50 years soil fumigation with methyl bromide and chloropicrin has been an indispensable tool because of their broad-spectrum and great efficacy (Martin, 2003). The Montreal Protocol for methyl bromide, the EU phase-out of chloropicrin on June 2013 (European Directive ECC 91/414) and re-evaluation of pesticide usage in the worldwide has dramatically reduced the arsenal of available fumigants and/or limited their use. Therefore, some soil-borne diseases, including Verticillium wilt of chrysanthemum, have become more difficult to manage.

In the last 20 years, there have been significant efforts in searching for alternative soil treatments (Martin, 2003). One of the alternatives to methyl bromide or chloropicrin is the use of dimethyl disulfide (DMDS), actually under evaluation for registration as a new soil fumigant in EU (Zanon *et al*., 2014a). DMDS is present in several natural sources, especially in plants belonging to the Alliaceae and Brassicaceae families (Arnault *et al*., 2013). This volatile compound is common in the global sulphur cycle and detected as a metabolite in several biological processes (Ruzo, 2006).

DMDS has shown good control of several soil-borne fungal plant pathogens in trials conducted in France, Italy, Spain, Israel, China and California. (Gerik, 2005; Rosskopf *et al*., 2005; Garibaldi *et al*., 2008; Gamliel *et al*., 2009; Heller *et al*., 2009; Cabrera *et al*., 2014; Li *et al*., 2014; Gómez-Tenorio *et al*., 2015). DMDS has zero ozone depletion potential (ODP), and is characterized by a favourable toxicological and environmental profile (Auger and Charles, 2003). In addition, DMDS has been reported as a systemic resistance elicitor in different plants (Huang *et al*., 2012). The efficacy of DMDS in disease control resulted in enhancement of plant growth and the marketable yield of potatoes, strawberries and tomatoes growing in DMDS-fumigated soil (Coosemans, 2005; Fritsch, 2005; Heller *et al*., 2009; Meldau *et al*., 2013).

The aim of the present study was to evaluate the efficacy of DMDS as pre-plant treatment against *V. dahliae* and its impact on chrysanthemum yield.

2. Materials and methods

Trial conditions, experimental design and statistical analysis of data

Dimethyl disulfide (DMDS), as EC (94.1%) and AL (99.1%) formulation, was applied to control the vascular wilt fungus *Verticillium dahliae* in greenhouse experiments conducted in the two successive seasons of 2013 and 2014. The trials were carried out in two grower fields located at Montecarlo (Lucca) (lat. 43°86'N, long. 10°68'W) Tuscany (Italy). The site is one of the major chrysanthemum production areas in Italy.

The soil of the fields was loamy and naturally infected by *V. dahliae* with organic matter content between 1.26% and 1.35%, and a pH between 7.15 and 7.20. The experimental site had a history of several chrysanthemum crops during the seasons prior to the beginning of this study. In the previous year's chrysanthemum crop, a severe disease outbreak caused by *V. dahliae* was reported by local extension services and confirmed by laboratory analysis.

In 2013, DMDS was applied at the rate of 600 kg ha^{-1} by shank application (Accolade/Paladin® AL) and was compared with chloropicrin (CP, 500 kg ha^{-1}) and non-treated control.

In 2014 DMDS was applied at the same rate, 600 kg ha^{-1} of a.i., via drip-irrigation (Accolade/Paladin® EC), and was compared with non-treated controls and Metam-sodium (Metam-Na, 1400 kg ha^{-1}) because chloropicrin phased out at the end of June 2013. Different formulations were used to compare their efficacy against the same target pathogen; drip-irrigation in the second year was included considering the sustainability of fumigant drip use in protected crops in EU (Ajwa *et al*., 2003; Ajwa and Trout, 2004). This assumption, beside the same dose rate of a.i. used and very similar formulations (94.1 vs. 99.1%) used in the trials, was based on previous published data where DMDS was successfully applied against several soil-borne pathogens both in drip (Garibaldi *et al*., 2008; Gamliel *et al*., 2009; Cabrera *et al*., 2014; Gómez-Tenorio *et al*., 2015) and shank (Gilardi *et al*., 2015; Myrta, unpublished) applications.

Treated plots were covered for 2 weeks with a 0.03 mm virtually impermeable plastic film (VIF). After film removal, the bare soil was left to aerate for one week to allow ventilation and gas dissipation and then chrysanthemum rooted cuttings cultivar 'Veneri' were transplanted in each plot. 'Veneri' was used in the trials since it has been considered one of the most *V. dahliae*-susceptible cultivar and one of the best commercial cultivar in the region. Transplants were confirmed free of *V. dahliae* by randomly testing fifty cuttings before planting as described in the following sub-chapter. Trials were arranged in a randomized complete block design and each treatment was replicated three times. Conventional crop management was followed as recommended in the region for chrysanthemum production in greenhouse conditions.

Verticillium wilt was recorded visually during the second half of the growing season by counting the number of symptomatic chrysanthemum plants. In the middle of the plots, to minimize possible influence from adjacent treatments, plants (n = 30) were randomly chosen and analysed for each replicate of each treatment. Plots were 36 m long and 4 m wide with 36 plant rows.

The incidence of Verticillium wilt was expressed as cumulative percentage of diseased plants recorded up to the end of the experiments. The yield of marketable chrysanthemum stems in the trial plots was also recorded. Percentage data were transformed (arcsine square root) prior to analysis of variance. When the F statistic of an ANOVA was significant at $P < 0.05$, means were compared with Fischer's least significant difference (LSD) test at the 5% significance level. Statistical analyses were performed using CoStat 6.4 (Cohort Software, Monterey, CA , USA).

Determination of Verticillium wilt in the field

At the end of the crop cycle, when asymptomatic flowering stems of chrysanthemum were harvested leaving the basal part of cut stems (residual stems, ca. 5-10 cm) in the ground, symptomatic plants from each plot were collected for diagnosis. Furthermore, in order to verify the presence of *V. dahliae* in asymptomatic harvested plants, 10 residual stems were collected and analysed for each plot.

Wilted plant stems and residual stems (separated from collar roots) were washed under running tap water, surface sterilized for 10 min in an aqueous solution containing sodium hypochlorite (NaOCl) (1% available chlorine) and ethanol (50% v/v), rinsed twice in sterile distilled water, and dried in a laminar flow-cabinet.

After surface disinfection, wilted plant portions and 5 serial sections (1 cm) of each residual stem (starting from ground level) were placed on potato dextrose agar (PDA) (Difco, Detroit, MI, USA) supplemented with 300 mg l^{-1} streptomycin sulfate and incubated for 7 days at 24°C. Plates were periodically inspected to detect the presence of *V. dahliae* characteristic colonies. If necessary, the diagnosis was confirmed by microscopic analysis.

3. Results

Verticillium wilt incidence

In the growing season 2013, vascular wilt incidence in the untreated plots was 52% and soil fumigation with DMDS or CP significantly reduced the percentage of diseased plants to less than 1%.

In the experiment conducted in 2014, incidence of *V. dahliae* in control plots was 27%. DMDS or Metam-Na application provided significant reduction of Verticillium wilt incidence with a percentage of diseased plants equal to 0 and 1.67 respectively (Fig. 1).

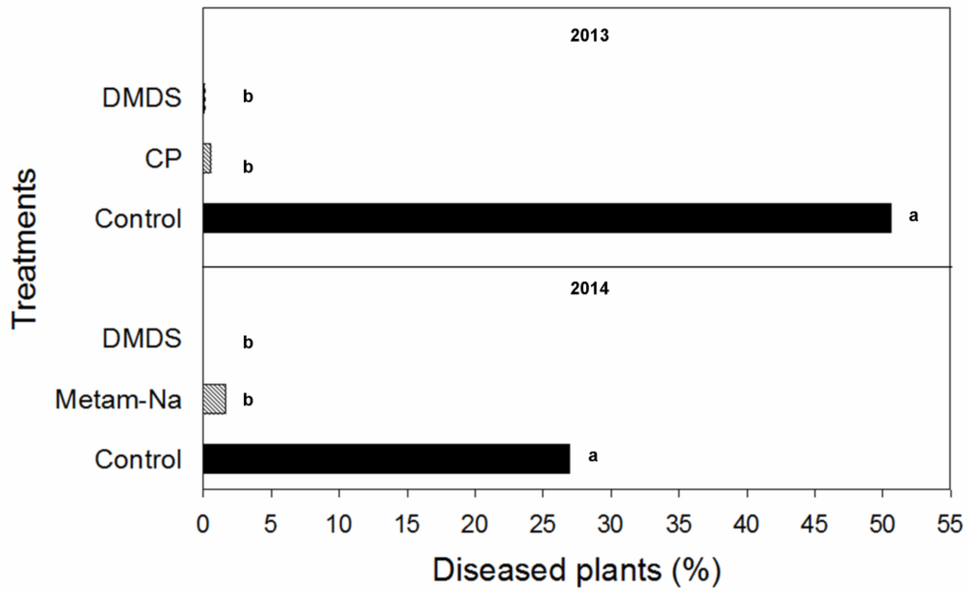

Fig 1. Verticillium wilt incidence (%) in chrysanthemum cv. ‘Veneri’ plants in greenhouse trials in 2013 and 2014. Treatments followed by the same letter are not significantly different according to the LSD test (P = 0.05). Data were arcsine square root-transformed before ANOVA, but untransformed means are presented. DMDS = Dimethyl disulfide; CP = Chloropicrin; Metam-Na = Metam sodium; Control = no treatment.

In the experiments conducted in both 2013 and 2014, fumigation treatments with DMDS, CP or Metam-Na exhibited the highest number of marketable chrysanthemum stems, which was significantly higher than those from untreated control plots. In the growing season 2013, the average number of marketable chrysanthemum stems in the DMDS and CP fumigated plots was 40 and 39 respectively, whereas it was 29 in the untreated control plots. In 2014, the average number of marketable chrysanthemum stems in the DMDS and Metam-Na treated plots was 45 and 44 respectively but it was 35 in the untreated control plots (Fig. 2).

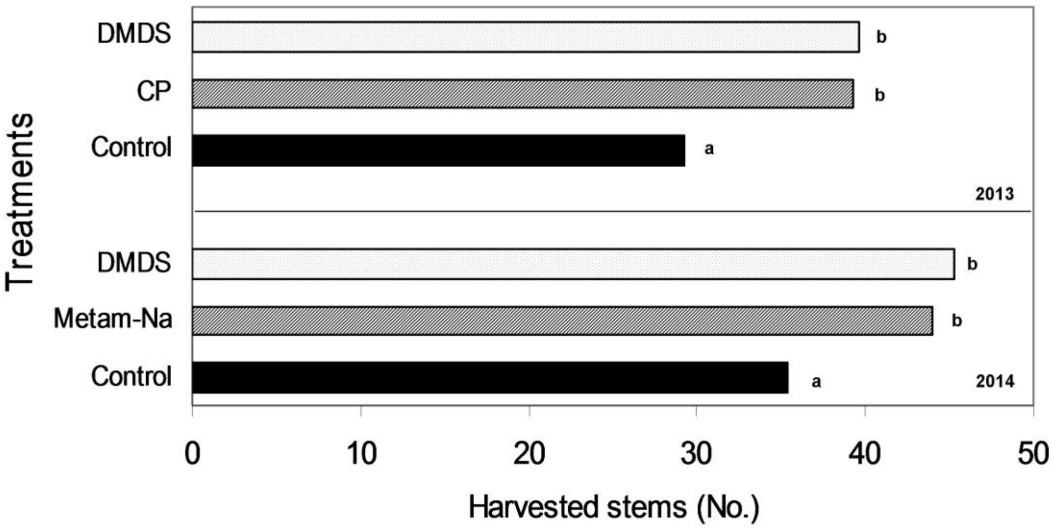

Fig. 2. Average number of marketable chrysanthemum stems cv. 'Veneri' yielded in the growing seasons 2013 and 2014. Plants (n = 30) were randomly chosen and analysed for each replicate of each treatment. Means followed by the same letter are not significantly different according to the LSD test (P = 0.05). DMDS = Dimethyl disulfide; CP = Chloropicrin; Metam-Na = Metam sodium; Control = no treatment.

In both 2013 and 2014 growing seasons, serial sections from residual stems of asymptomatic harvested chrysanthemum plants revealed the presence of *V. dahliae* throughout much of the stem tissues in control plots. In some cases, there were 5 contiguous sections infected by *V. dahliae* although the first section showed the prevalent presence of the pathogen that gradually decreased in the contiguous upward sections. Therefore the pathogen was isolated also from symptomless tissues (Fig. 3).

Treatments significantly reduced the presence of the pathogen in residual stems. In the growing season 2013, none of the sections collected form residual stems in plots treated with DMDS or CP was infected by *V. dahliae.* In 2014, DMDS treatment gave the lowest number of infected sections (first position) whereas some residual stems derived from plots treated with Metam-Na showed infected sections until the third position.

V. dahliae was not isolated from the chrysanthemum rooted cuttings before planting but it was isolated from all wilted plant stems at the end of the crop cycle.

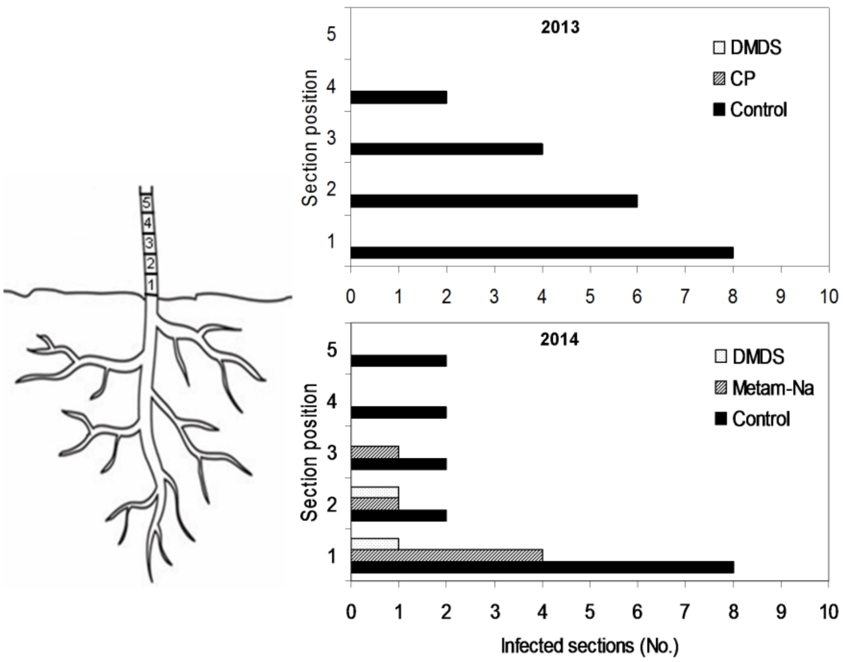

Fig. 3. Presence of *V. dahliae* in sections of asymptomatic chrysanthemum residual stems after crop harvest. In 2013, none of the sections collected form residual stems in plots treated with DMDS or CP was infected by *V. dahliae.* DMDS = Dimethyl disulfide; CP = Chloropicrin; Metam-Na = Metam sodium; Control = no treatment.

4. Discussion

Efficacy of DMDS fumigation was evaluated against *Verticillium dahliae* on chrysanthemum in greenhouse trials. The experimental results showed that fumigation with DMDS provided significant reduction of Verticillium wilt comparable with other commonly used chemical fumigants (CP in 2013 and Metam-Na in 2014) in naturally infected soils. In our knowledge this is the first report on the efficacy of DMDS alone against chrysanthemum Verticillium wilt.

Furthermore, the number of marketable chrysanthemum stems was consistently increased with the application of DMDS compared with non-treated control and was equal to the other standard chemical fumigants used in both growing seasons 2013 and 2014.

Dimethyl disulfide used alone showed a good activity at high dosages (800 kg ha^{-1}) against *V. dahliae* on grafted eggplants in naturally infected soils (Gilardi *et al*., 2010) and strawberry in artificial and natural infected soils (Fritsch, 2005).

Usually chrysanthemum is grown year after year without rotation. This intensive greenhouse crop system creates optimal conditions for the development and multiplication of soil-borne pathogens such as *V. dahliae*. Moreover, farmers routinely incorporate chrysanthemum residues in the soil to improve soil fertility, and this too increases the amount of pathogens inoculum in the soil.

This study shows that *V. dahliae* was not only isolated from diseased, but also from symptomless tissues in residual chrysanthemum stems. Anyway DMDS, as well as CP and, in a lesser extent, Metam-Na chemical fumigation treatments reduced the presence of the pathogen in residual stems of asymptomatic harvested chrysanthemum.

This effect, although apparently not commercially relevant as it doesn't affect crop yield, it is epidemiologically important and can play a relevant role in the disease management in a long term. Formation of microsclerotia on dying host debris in the soil can cause an increase of inoculum density in the following year, especially if the increase is greater than the reduction in microsclerotia due to mortality (Mol *et al*., 1995). A lower rate of infected peanut and bean crop residues resulted in a lower *V. dahliae* microsclerotia population in subsequent crops (Hoekstra, 1989; Chawla *et al*., 2012).

Results from the present study suggest the importance of removing chrysanthemum residues infected with *V. dahliae*, and the effectiveness of DMDS fumigation as well as CP and Metam-Na in decreasing *V. dahliae* residual stems colonization, both addressing the need to lower pathogen inoculum density in soil.

Doran and Parkin (1996) indicated that soil health is inseparable from issues of sustainability. Therefore, DMDS in EU could be a new effective solution to control Verticillium wilt of chrysanthemum. Thanks to its new mechanism of action, after its registration, it can be considered in pathogen control sustainable programmes in floriculture. This will become very important for the sector when key fungicide fumigants are actually not registered (Chloropicrin) or limited in their use (only once every 3 years, Dazomet and Metam-Na). DMDS is under evaluation in a large-scale IPM trial programme, in combination with biological products and soil solarization (Basallote-Ureba *et al*., 2010; Zanon *et al*., 2014b; Myrta and Santori, unpublished).

Conflict of interest

S. Pecchia had a partial financial support from Certis Europe B.V. for the submitted work; A. Myrta and A. Santori are Certis Europe B.V. employees. No other relationships or activities that could appear to have influenced the submitted work are declared.

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Highlights

Efficacy of the new soil fumigant dimethyl disulfide (DMDS) was studied in 2013–2014.

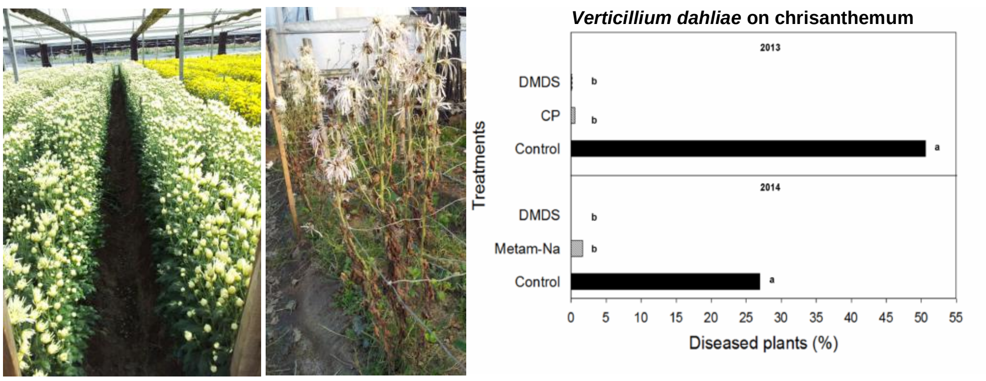
Accolade/Paladin® was assessed to control *Verticillium dahliae* on chrysanthemum.

DMDS decreased significantly Verticillium wilt incidence in the treated plots.

DMDS increased significantly the number of marketable chrysanthemum stems.

DMDS decreased the presence of *V. dahliae* in asymptomatic residual stems.

Graphical abstract



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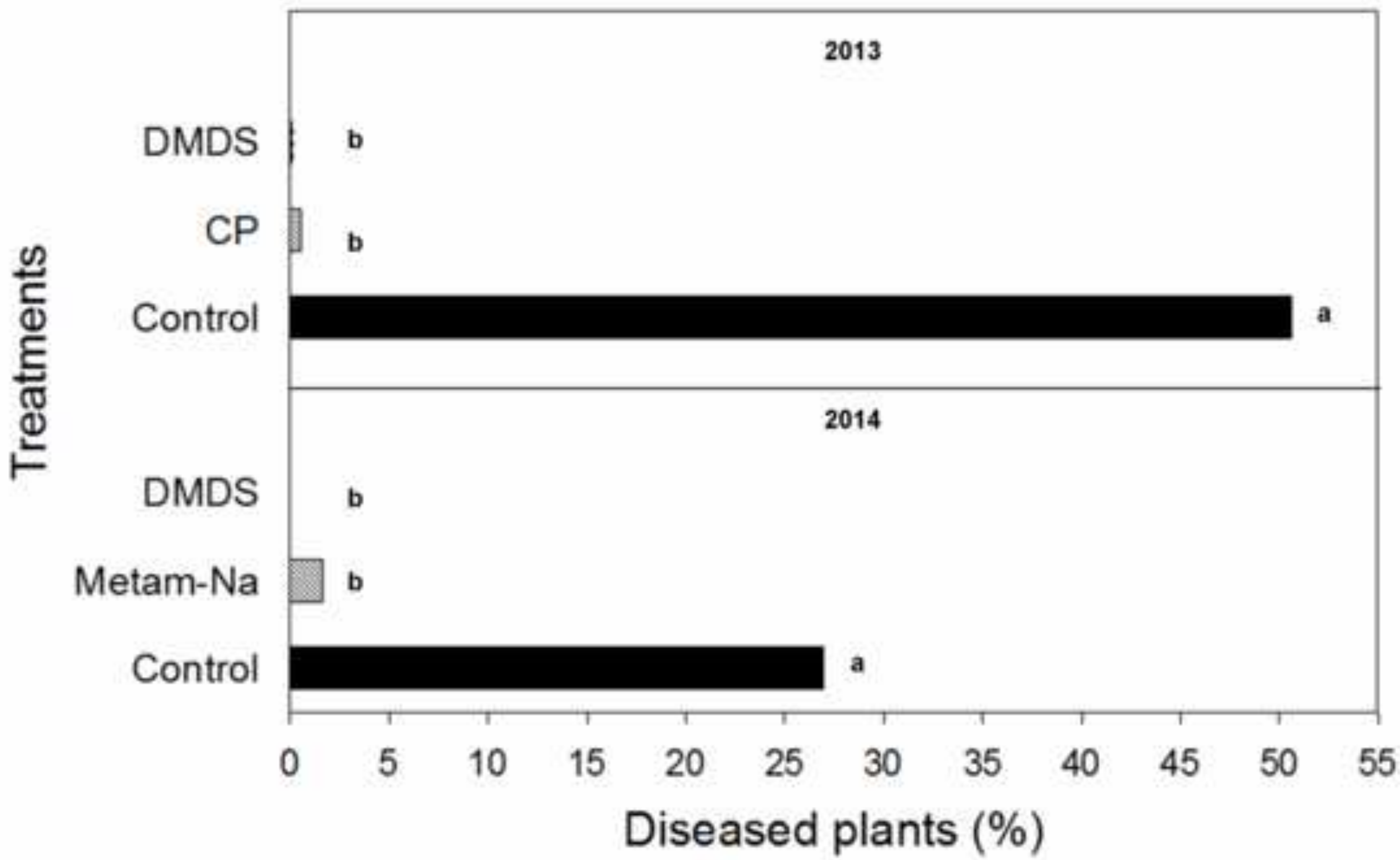

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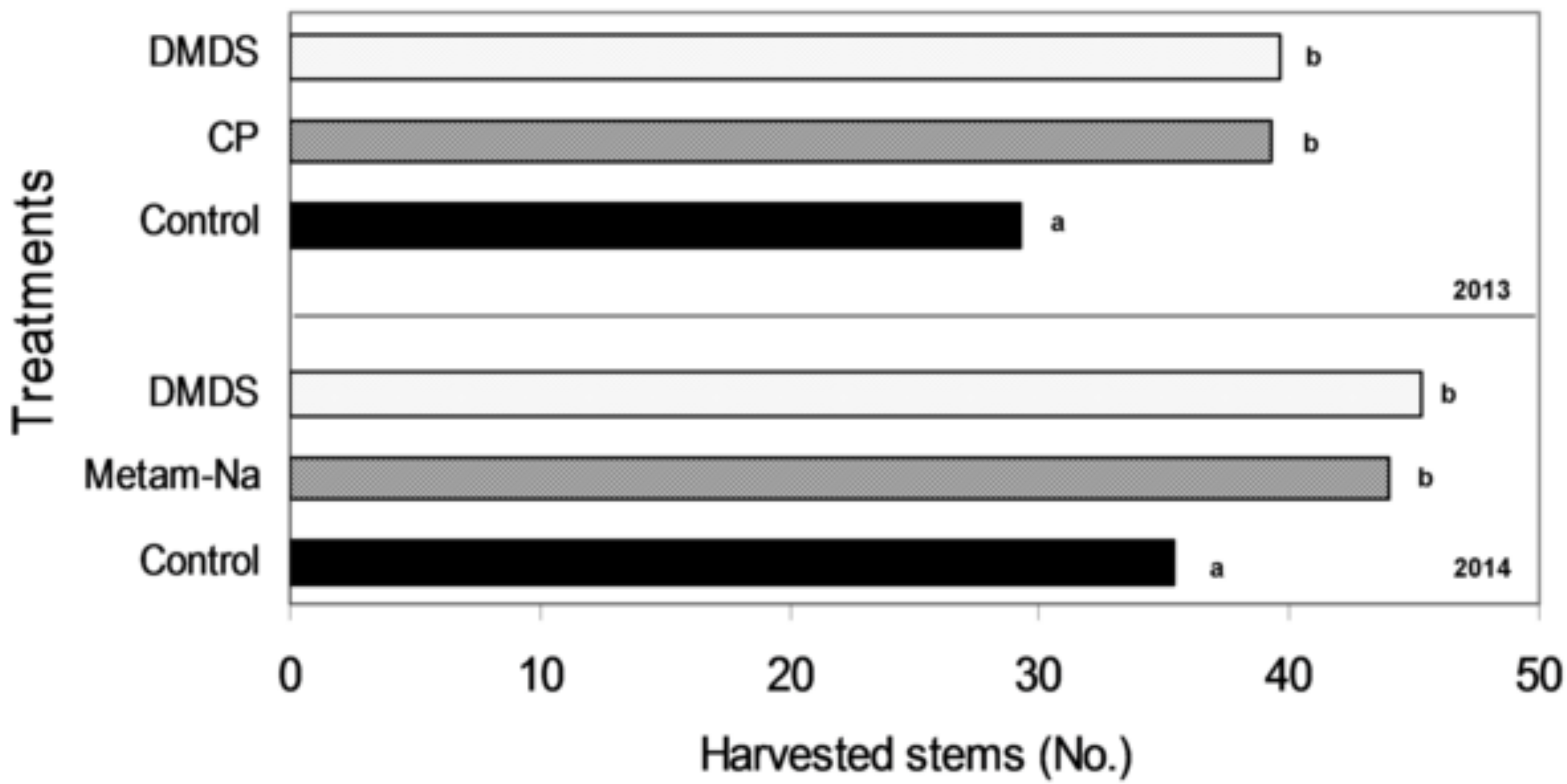

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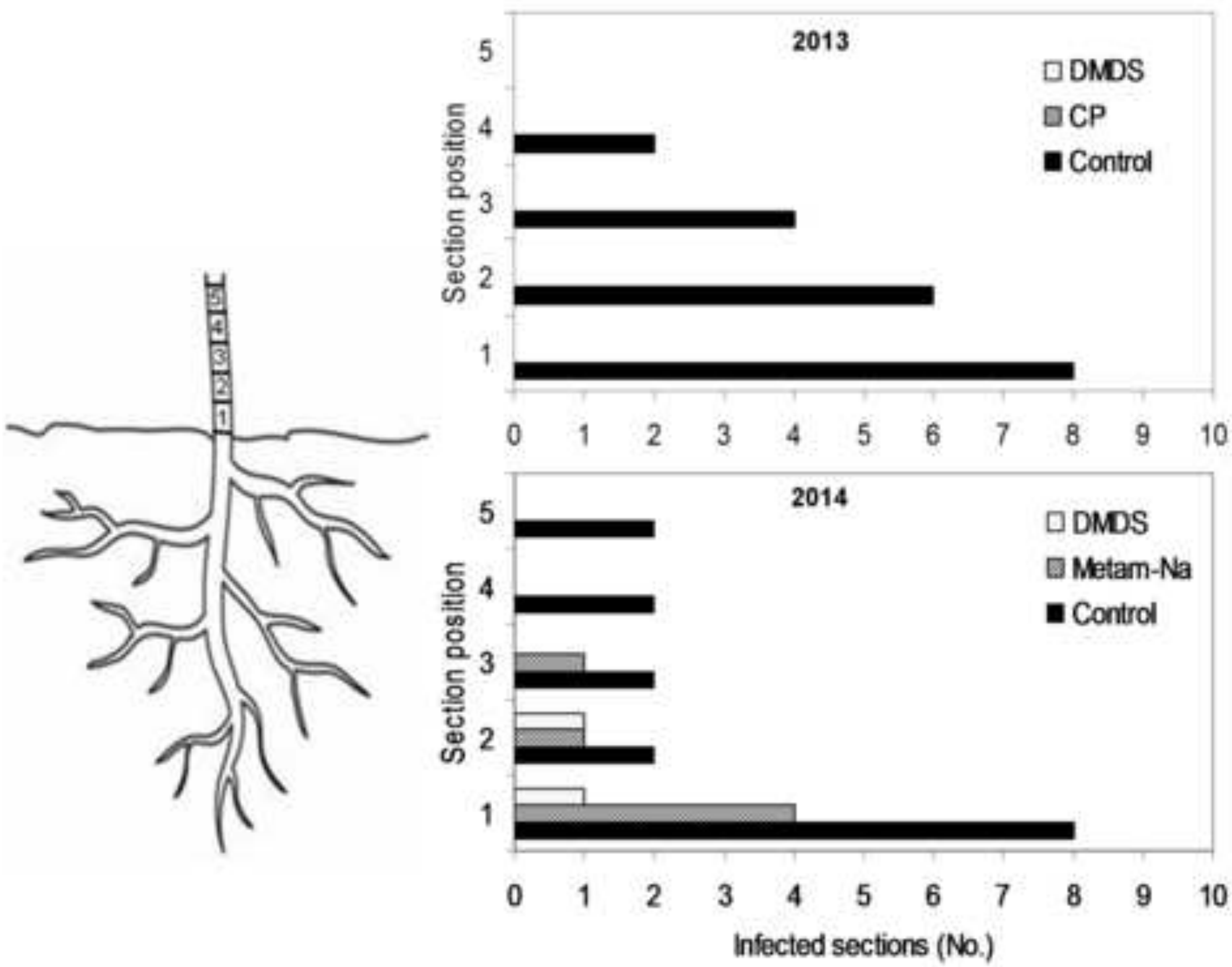

Captions

Fig 1. Verticillium wilt incidence (%) in chrysanthemum cv. 'Veneri' plants in greenhouse trials in 2013 and 2014. Treatments followed by the same letter are not significantly different according to the LSD test (P = 0.05). Data were arcsine square root-transformed before ANOVA, but untransformed means are presented. DMDS = Dimethyl disulfide; CP = Chloropicrin; Metam-Na = Metam sodium; Control = no treatment.

Fig. 2. Average number of marketable chrysanthemum stems cv. 'Veneri' yielded in the growing seasons 2013 and 2014. Plants (n = 30) were randomly chosen and analysed for each replicate of each treatment. Means followed by the same letter are not significantly different according to the LSD test (P = 0.05). DMDS = Dimethyl disulfide; CP = Chloropicrin; Metam-Na = Metam sodium; Control = no treatment.

Fig. 3. Presence of *V. dahliae* in sections of asymptomatic chrysanthemum residual stems after crop harvest. In 2013, none of the sections collected form residual stems in plots treated with DMDS or CP was infected by *V. dahliae.* DMDS = Dimethyl disulfide; CP = Chloropicrin; Metam-Na = Metam sodium; Control = no treatment.

***Highlights (for review)**

Efficacy of the new soil fumigant dimethyl disulfide (DMDS) was studied in 2013–2014.

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Graphical Abstract