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1 **Pathogenicity of *Phytophthium chamaehyphon*: a new player in Kiwifruit Vine Decline**
2 **Syndrome of *Actinidia chinensis* var. *deliciosa* (A. Chev.) A. Chev. ‘Hayward’ in Italy**

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15 **Abstract**

16 Kiwifruit Vine Decline Syndrome (KVDS) is a serious soil-borne disease that degrades the fine roots
 17 of both *Actinidia chinensis* var. *deliciosa* and var. *chinensis*. The disease seems to be the result of an
 18 interaction between several soil-borne pathogens, mostly oomycetes, and waterlogging. This work
 19 investigates the pathogenicity of the oomycete *Phytophthora chamaeaphon* recently isolated from
 20 roots of diseased plants. Pathogenicity was tested in 6-month-old and 1-year-old plants that after
 21 inoculation were flooded up to three times to induce symptom appearance. Leaf wilting and root rot
 22 typical of KVDS was observed in all the plants inoculated with *P. chamaeaphon* strain KD-15
 23 (PCHA) and in all the positive controls potted in a mix of peat and soils collected in KVDS-affected
 24 orchards, while negative controls remained symptomless. Disease development on 6-months-old
 25 plants was characterized by unusual degradation of the not-lignified collar, occurring even in absence
 26 of flooding. Conversely, on 1-year-old plants, symptoms faithfully reproduced KVDS dynamics
 27 observed in orchard. This work confirmed the pathogenicity of *P. chamaeaphon* and raised new
 28 questions about the actual role of waterlogging in KVDS etiology.

29

30 **Keywords:** Kiwifruit Vine Decline Syndrome, oomycetes, waterlogging, pathogenicity test, root
 31 rot, collar rot

32

33 Italy is one of the major kiwifruit producer worldwide, satisfying by itself over 15% of the global
 34 market demand (FAOSTAT, 2020). A new disease known as Kiwifruit Vine Decline Syndrome
 35 (KVDS) has been reported in Italy since 2012 and currently affects 25% (6,160 ha) of Italian kiwifruit
 36 production area (Tacconi et al. 2020). KVDS mainly attacks the softer root tissues (fine roots) causing
 37 sudden and fast wilting of the canopy, usually visible after the first heat waves occur in late-spring
 38 and early-summer (June-August) (Tacconi et al. 2015; Savian et al. 2020a, b; Bardi 2020). KVDS
 39 etiology has not been fully clarified, but it seems the result of an interaction between waterlogging
 40 and soil-borne pathogens (Savian et al. 2020a). In particular, oomycetes (*Phytophthora* and

41 *Phytophthora* genera) seem to play a major role in KVDS (Savian et al. 2020a; Donati et al. 2020;
 42 Prencipe et al. 2020), however, also fungi (genus *Desarmillaria*) and bacteria (genus *Clostridium*)
 43 have been associated with the disease (Donati et al. 2020; Spigaglia et al. 2020).

44 The role of the interaction between soil-borne pathogens and waterlogging in the development of
 45 KVDS was demonstrated using soils from infected orchards of Friuli Venetia Giulia (FVG, NE Italy;
 46 Savian et al. 2020a). Two species, belonging to *Phytophthora* genus, were isolated and emerged as
 47 potential pathogens: *Phytophthora vexans* and *Phytophthora chamaeaphon*. Pathogenicity of *P.*
 48 *vexans* on kiwifruit vine and its association to KVDS was previously demonstrated by Prencipe et al.
 49 (2020), while the fulfillment of Koch's postulates for *P. chamaeaphon* was not determined and is
 50 the focus of the present work.

51 *P. chamaeaphon* was also isolated in Piedmont (NW Italy) from kiwifruit vines showing typical
 52 KVDS symptoms, in the framework of an extensive field monitoring of 18 orchards during 2016-
 53 2019 seasons. Root sampling and isolation were performed according to Prencipe et al. (2020). One
 54 representative isolate, grown on V8 agar, was observed morphologically according to de Cock et al.
 55 (2015). Colonies showed typical mycelia of a *Pythium* species, and older cultures showed sub-globose
 56 sporangia, ($20.19 \pm 0.36 \mu\text{m}$), containing circular shaped zoospores (9–10 μm). DNA was extracted
 57 from isolates using Omega E.Z.N.A. Fungal DNA Mini Kit (VWR, USA), according to
 58 manufacturer's instructions. Species identification was confirmed by sequencing ITS region using
 59 primers ITS1/ITS4 (White et al. 1990), cytochrome oxidase I region (COI) using
 60 FM85mod/OomCOILevup primers (Robideau et al. 2011), and the large subunit (LSU) rDNA using
 61 NL1/NL4 primers (Baten et al. 2014). Sequences obtained from field isolate shared 99% identity for
 62 ITS region and 100% identity for LSU and COI with *P. chamaeaphon* isolates from Savian et al.
 63 2020a.

64 *P. chamaeaphon* strain KD-15 from FVG (ITS region sequence under the accession number
 65 MN535819; Savian et al. 2020a), further identified based on sequencing the LSU and COI regions
 66 (accession numbers MW431329 and MW430118), was selected for the pathogenicity test.

67 Pathogenicity was tested twice on 1-year-old (trial 1) and 6-month-old (trial 2) *Actinidia chinensis*
 68 var. *deliciosa* (A. Chev) A. Chev. ‘Hayward’ plants potted in sterilized perlite-peat mixture (1:2
 69 volumetric ratio). The oomycete was prepared according to Prencipe et al. (2020), growing *P.*
 70 *chamaehyphon* (PCHA) on wheat and hemp seeds for 7 days, and mixing the inoculum to the soil at
 71 a rate of 6 g/liter. Negative controls (NC) were inoculated with 6 g/l of autoclaved seed mixture, while
 72 positive controls (PC) pots were filled with infected soil and sterile peat to a volumetric proportion
 73 of 1:1. Infected soils were collected in June 2019, close to symptomatic roots from three orchards of
 74 Pordenone province (FVG, NE Italy) clearly affected by KVDS. Each thesis included 6 plants in trial
 75 1 and 12 plants in trial 2, potted on 6- and 3-liter vases, respectively. Both trials were kept in a
 76 greenhouse at 28±5°C. After transplanting, water content was maintained proximal to field capacity
 77 for 3 weeks for trial 1 and 4 weeks for trial 2, before applying three rounds of flooding and drainage
 78 to induce symptoms (Savian et al. 2020a). Wilting appearance was recorded after 25, 35, and 50 days
 79 post inoculation (dpi) in trial 1 and every 2-3 days in trial 2. To fulfil Koch’s postulates, re-isolations
 80 were performed from PCHA symptomatic plants and the isolates were molecularly identified by
 81 amplification and sequencing of the ITS region.

82 Typical wilting symptoms occurred in plants of PC and PCHA of both trials, while all NC plants
 83 remained symptomless until the end of the experiment (58 dpi).

84 On plants of trial 1, the disease progression was similar to that observed in the field, with wilting
 85 appearing only after waterlogging when the fine roots were heavily compromised. Wilting appeared
 86 after the third flooding cycle (50 dpi) where PCHA plants showed 68% symptomatic leaves, while
 87 PC plants showed 77% of symptomatic leaves (Figure 1). Re-isolation from PCHA plants fulfilled
 88 Koch’s postulate confirming the pathogenicity of *P. chamaehyphon*.

89 In trial 2 with younger plants, the symptoms evolution was much faster than in trial 1, probably
 90 because the infection took place in the non-lignified plant collar, causing a faster dieback. For this
 91 reason, not all the flooding cycles were applied to induce the disease (Figure 2). Wilting on PCHA
 92 plants appeared right after the end of the first flooding (30 dpi), and rapidly evolved to complete plant

93 decline for 92% of the plants (Figure 2). The disease progression in PC thesis was even faster: the
 94 plants started to wilt at 17 dpi, and one week before the first flooding cycle (24 dpi) 75% of the plants
 95 were dead, while the remaining ones died shortly after the first flooding application (Figure 2). Re-
 96 isolation from PCHA plants confirmed the Koch's postulate and the pathogenicity of *P.*
 97 *chamaehyphon* as observed for 1 year-old inoculated plants.

98 To the best of our knowledge, this is the first report of pathogenicity of *P. chamaehyphon* on kiwifruit.
 99 *Phytopythium* is a newly established genus including species formerly classified as *Pythium* species.
 100 This genus can inhabit both water and soil, and requires free water for the zoospore movement and
 101 for the initiation of the infection, as many other oomycetes (de Cock et al. 2015). Previously other
 102 oomycetes have been associated to KVDS: *Phytopythium vexans* (Prencipe et al. 2020), *Phytopythium*
 103 *helicoides* (Donati et al. 2020), *Phytophthora infestans* (Donati et al. 2020), *Phytophthora*
 104 *citrophthora* and *Phytophthora cryptogea* (Tacconi et al. 2015). It may be speculated that KVDS is
 105 enhanced if several pathogenic species are simultaneously infecting the roots, since the speed of
 106 symptom appearance and the disease incidence were higher in PC treatments compare to PCHA. Co-
 107 occurrence of *P. chamaehyphon* strain KD-15 and *Phytopythium vexans* was indeed observed in
 108 Savian et al. (2020) with a frequency of 55%. However, although the concept of a "pathobiome"
 109 associated with KVDS is well accepted (Savian et al. 2020a; Donati et al. 2020; Prencipe et al. 2020;
 110 Spigaglia et al. 2020), we do not fully know yet the composition of the KVDS microbial community
 111 and the interactions occurring between these pathogens.

112 Our findings confirm some preliminary indications suggesting that pathogens aggressiveness is
 113 enhanced when waterlogging is applied (Savian et al. 2020a). Indeed, the application of the flooding
 114 cycle accelerated the disease development in both PC and PCHA plants, while those in NC remain
 115 symptomless (Figure 1). However, the high mortality rate of 6-month-old plants, observed before
 116 waterlogging application (24 dpi) in PC plants, permits to hypothesize that KVDS might develop not
 117 only during/after flooding, but also at soil water content proximal to field capacity. Furthermore, the
 118 sudden appearance of symptoms on 6-month-old PCHA plants, right after the first day of flooding,

119 indicates that the infection had started before the flooding application. Previously, Donati et al. (2020)
120 already showed that the disease could be observed also in plants maintained at 70% of the estimated
121 field capacity, without flooding. Moreover, in the study of Savian et al. (2020a), the leaf/root ratio of
122 plants in unsterile soil without flooding was found more unbalanced than that of plants in sterile soil
123 with flooding. KVDS being active even at soil water content proximal to field capacity could explain
124 the reason why agronomical practices so far implemented to control the disease (tensiometer-driven
125 irrigation and raised beds) were only able to delay the appearance of the disease after replanting
126 (Tacconi et al. 2019). These results raise new questions on the effective role of root oxygen depletion
127 and consequently of strictly anaerobic microorganisms on the onset of the disease.

128 The symptoms on 1-year-old plants, normally used as propagation material, faithfully reflected the
129 disease development in orchard (Tacconi et al. 2015), while the uncommon rotting of the collar
130 observed on 6-month-old plants was not observed previously, but it is in accordance with KVDS
131 development, that usually degrades non-lignified tissues such as fine roots (Savian et al. 2020a).
132 Considering these results, plants with lignified collar should be preferred for pathogenicity tests
133 related to KVDS. In case only younger plants are available, modifications on the timing and duration
134 of flooding should be taken into consideration in order to control the symptom appearance.

135 In conclusion, this work clearly demonstrated the pathogenicity of *P. chamaehyphon* and provided
136 indication regarding the planting material to conduct KVDS-related tests. Furthermore, knowing that
137 KVDS can develop also at soil water content proximal or inferior to field capacity may have a
138 significant impact on both disease etiology and management strategies.

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Figure 1. Kiwifruit Vine Decline Syndrome (KVDS) symptoms observed during the pathogenicity test. Negative control (NC) plants inoculated with sterile seed mixture are in a) and d); plants infected with *P. chamaeophyon* strain KD-15 (PCHA) are in b) and e); Positive control (PC) plants inoculated with soil collected from KVDS affected orchards are in c) and f). Wilting symptoms are shown for: 1-year-old plants at the end of the experiment in a-c); 6-month-old plants at 24, 28-31 and 45 dpi in d-f). On the right, the roots of 6-month-old plants at the end of the experiment are shown: g) healthy roots of NC plants; h-k) symptoms on the roots of PCHA and PC plants. h-i) typical symptoms of KVDS on feeding roots, red circles in i) indicate the areas selected for re-isolation. j-k) atypical rots observed on the collar.

Figure 2. Percentage of plants with Kiwifruit Vine Decline Syndrome (KVDS) symptoms (disease incidence) during the pathogenicity test in 1-year-old and 6-month-old plants. Plants with KVDS symptoms are colored in orange while asymptomatic plants are in green. Shaded area indicates the application and duration of the flooding periods. NC, plants inoculated with sterile seed mixture; PCHA, plants inoculated with *P. chamaeophyon* strain KD-15; PC, plants potted on soil collected from KVDS affected orchards.