A survey on the infection of Onion yellow dwarf virus and Iris yellow spot tospovirus in seed and bulb productions systems of onion in Calabria, Italy

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5	Corresponding Author	Family Name	**Albanese**
6		Particle	
7		Given Name	**Giuliana**
8		Suffix	
9		Organization	Università degli Studi Mediterranea di Reggio Calabria
10		Division	Department of AGRARIA
11		Address	Feo di Vito, Reggio Calabria 89122, Italy
12		e-mail	galbanese@unirc.it
13	Author	Family Name	**Manglli**
14		Particle	
15		Given Name	**Ariana**
16		Suffix	
17		Organization	Università degli Studi Mediterranea di Reggio Calabria
18		Division	Department of AGRARIA
19		Address	Feo di Vito, Reggio Calabria 89122, Italy
20		Organization	Consiglio per la Ricerca in agricoltura e l'analisi dell'Economia Agraria, Research Centre for Plant Protection and Certification
21		Division	
22		Address	Via C.G. Bertero 22, Rome 00156, Italy
23		e-mail	
24	Author	Family Name	**Tomassoli**
25		Particle	
26		Given Name	**Laura**
27		Suffix	

28		Organization	Consiglio per la Ricerca in agricoltura e l'analisi dell'Economia Agraria, Research Centre for Plant Protection and Certification
29		Division	
30		Address	Via C.G. Bertero 22, Rome 00156, Italy
31		e-mail	
32	Author	Family Name	**Tiberini**
33		Particle	
34		Given Name	**Antonio**
35		Suffix	
36		Organization	Università degli Studi Mediterranea di Reggio Calabria
37		Division	Department of AGRARIA
38		Address	Feo di Vito, Reggio Calabria 89122, Italy
39		e-mail	
40	Author	Family Name	**Agosteo**
41		Particle	
42		Given Name	**Giovanni E.**
43		Suffix	
44		Organization	Università degli Studi Mediterranea di Reggio Calabria
45		Division	Department of AGRARIA
46		Address	Feo di Vito, Reggio Calabria 89122, Italy
47		e-mail	
48	Author	Family Name	**Fontana**
49		Particle	
50		Given Name	**Anna**
51		Suffix	
52		Organization	Università degli Studi Mediterranea di Reggio Calabria
53		Division	Department of AGRARIA
54		Address	Feo di Vito, Reggio Calabria 89122, Italy
55		e-mail	
56	Author	Family Name	**Pappu**
57		Particle	
58		Given Name	**Hanu R.**
59		Suffix	
60		Organization	Washington State University
61		Division	Department of Plant Pathology
62		Address	Pullman 99164, WA, USA
63		e-mail	

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Abstract	A survey on the incidence of onion yellow dwarf virus (OYDV) and iris yellow spot tospovirus (IYSV) was carried out over three production cycles of onion 'Rossa di Tropea' in Calabria, Italy. OYDV was found to be the prevalent virus. 'Rossa di Tropea' seed adjacent to OYDV-infected green onion field had seedlings with 1.76% infection rate determining 36.2% and 98.67% infected plants in the bulbs and in the subsequent seed harvesting times, respectively. When seedbeds were at least one km away from other onion crops seedlings and bulb cultivation had the infection rate close to zero. OYDV was detected in whole plants except the roots and outer desiccated bulb skins. Seed transmission was not detected in 'Rossa di Tropea'. Early OYDV infection significantly reduced the number and weight of seeds/inflorescence compared to late season infection, while the weight of 100 seeds was not different in the two early and late OYDV infected plants. IYSV was never found in seedbeds. It was always detected first in seed crops (April) than in bulb crops (June), and the final infection rate was higher in seed (2.67%–3.33%) than in bulb crops (0%–0.87%), suggesting there was an internal source of viral inoculum in the field. IYSV was detected in 3/123 apex bulbs randomly collected from stored bulbs and in 12/12 apex fresh bulbs collected at harvest time from infected plants, showing the role of bulbs as IYSV inoculum source. On the contrary, randomly collected bulbs ($N = 109$) from warehouse and bulbs of infected plants ($N = 22$), transplanted after storage, did not result in IYSV-infected plants.

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A survey on the infection of *Onion yellow dwarf virus* and *Iris yellow spot tospovirus* in seed and bulb productions systems of onion in Calabria, Italy

Ariana Manglli · Laura Tomassoli · Antonio Tiberini · Giovanni E. Agosteo · Anna Fontana · Hanu R. Pappu · Giuliana Albanese

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Abstract A survey on the incidence of onion yellow dwarf virus (OYDV) and iris yellow spot tospovirus (IYSV) was carried out over three production cycles of onion 'Rossa di Tropea' in Calabria, Italy. OYDV was found to be the prevalent virus. 'Rossa di Tropea' seed adjacent to OYDV-infected green onion field had seedlings with 1.76% infection rate determining 36.2% and 98.67% infected plants in the bulbs and in the subsequent seed harvesting times, respectively. When seedbeds were at least one km away from other onion crops seedlings and bulb cultivation had the infection rate close to zero. OYDV was detected in whole plants except the roots and outer desiccated bulb skins. Seed transmission was not detected in 'Rossa di Tropea'. Early OYDV infection significantly reduced the number and weight of seeds/inflorescence compared to late season infection, while the weight of 100 seeds was not different in the two early and late OYDV infected plants. IYSV was never found in seedbeds. It was always detected first in seed crops (April) than in bulb crops (June), and the final infection rate was higher in seed (2.67%–3.33%) than in bulb crops (0%–0.87%), suggesting there was an internal source of viral inoculum in the field. IYSV was detected in 3/123 apex bulbs randomly collected from stored bulbs and in 12/12 apex fresh bulbs collected at harvest time from infected plants, showing the role of bulbs as IYSV inoculum source. On the contrary, randomly collected bulbs ($N = 109$) from warehouse and bulbs of infected plants ($N = 22$), transplanted after storage, did not result in IYSV-infected plants.

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A. Manglli · A. Tiberini · G. E. Agosteo · A. Fontana · G. Albanese (✉)
Department of AGRARIA, Università degli Studi Mediterranea di Reggio Calabria, Feo di Vito, 89122 Reggio Calabria, Italy
e-mail: galbanese@unirc.it

A. Manglli · L. Tomassoli
Consiglio per la Ricerca in agricoltura e l'analisi dell'Economia Agraria, Research Centre for Plant Protection and Certification, Via C.G. Bertero 22, 00156 Rome, Italy

H. R. Pappu
Department of Plant Pathology, Washington State University, Pullman, WA 99164, USA

Introduction

Onion (*Allium cepa* L.) is the most widely cultivated species of the genus *Allium*, family *Amaryllidaceae*. The production of onion in Italy was of 410,535 tons in 2017 on 12,248 ha (ISTAT 2017) including many cultivars/biotypes of high organoleptic quality. In Calabria, southern Italy, onion production was about 34,700 tons cultivated on 922 ha (ISTAT 2017). In this region, 'Rossa di Tropea' onion holds a Protected Geographical Indication (PGI) trademark. Soil and climatic characteristics for the cultivation of 'Rossa di Tropea' extend

through the provinces of Catanzaro, Cosenza, and Vibo Valentia, and confer to this cultivar its distinctive organoleptic properties that are highly appreciated in Italy and abroad where exported.

A large number of diseases caused by bacteria, fungi, and viruses affect onion. The viral species known to infect onion belong to the genera *Allexivirus*, *Carlavirus*, *Potyvirus,* and *Orthotospovirus.* Among them, potyviruses and orthotospoviruses induce the most important damage to this crop, whereas allexiviruses and carlaviruses cause latent infections, but cause perceptible damage in mixed infection with potyviruses (Katis et al. 2012).

Amongst members of the genus *Potyvirus,* the species *Onion yellow dwarf virus* (OYDV) is the most economically important virus affecting *Allium* species (Van Dijk 1993), especially onion and garlic. OYDV is transmitted in a non persistent manner by more than 50 aphid species (Drake et al. 1933), and *Myzus persicae* (Sulzer) is reported as the most efficient vector, followed by *Aphis craccivora* (Koch) and *A. gossypii* (Glover) (Abd El-Wahab 2009; Kumar et al. 2011). OYDV has a host range limited to *Allium* species and is detectable in almost all onion and garlic growing regions of the world (Dovas et al. 2001; Pappu et al. 2005; Katis et al. 2012; Chodorska et al. 2014; Majumder and Johari 2014; Vončina et al. 2016; Majumder et al. 2017; Sivaprasad et al. 2017). In Italy, OYDV was first reported based on symptoms on onions (Marani and Bertaccini 1983); the virus was later confirmed by molecular assays (Dovas and Volvas 2003), and 100% of infection in 'Rossa di Tropea' onion was reported in Calabria (Parrella et al. 2005). OYDV causes severe economic losses both in onion seed and bulb crops (Hoa et al. 2003; Elnagar et al. 2011; Kumar et al. 2012). The virus remains persistent in onion sprouts, plant residues and bulbs (Schwartz and Mohan 2008; Katis et al. 2012) whereas seed transmission was reported only in a few local cultivars (Ibrahim et al. 1996; Abd El-Wahab et al. 2009).

Three species of orthotospoviruses are able to naturally infect onion: *Impatiens necrotic spot tospovirus* (INSV), *Iris yellow spot tospovirus* (IYSV), and *Tomato spotted wilt tospovirus* (TSWV) (Mullis et al. 2004; Vicchi et al. 2008; Pappu et al. 2009). IYSV was first identified in *Iris hollandica* (Cortês et al. 1998). IYSV became an important pathogen that causes severe disease in onion worldwide, especially in North and South America (Pozzer et al. 1999; Crowe and Pappu 2005; Gent et al. 2006; Hoepting et al. 2007, 2008; Bag et al. 2015). The virus is transmitted by *Thrips tabaci* Lindeman (Cortês et al. 1998; Nagata et al. 1999; Krizman et al. 2001) and less efficiently by *Frankliniella fusca* Hinds (Srinivasan et al. 2012). In the Mediterranean basin, IYSV was reported in Spain (Cordoba-Selles et al. 2005; Muñoz et al. 2014), France (Huchette et al. 2008), and Italy (Tomassoli et al. 2009; Manglli et al. 2012; Turina et al. 2012). The virus is not seed-transmitted (Krizman et al. 2001; Bulajić et al. 2009), but bulb transmission was suggested (Robène-Soustrade et al. 2006; Schwartz 2008; Weilner and Bedlan 2013), although the presence of the virus in bulbs has not been reported by other authors (Kritzman et al. 2001; Boateng and Schwartz 2013).

In this study, the incidence of OYDV and IYSV was investigated during three production cycles (2012–2015) in Calabria aiming to develop effective management practices to ensure better yields of 'Rossa di Tropea'. Due to the different features of these two viruses, two parallel and independent approaches were used, starting from surveys to assess the incidence of OYDV and IYSV in different production phases by visual inspection and laboratory assays. Moreover, for OYDV, some disease aspects such as virus presence in various plant parts, and virus transmission through seed in cv. Rossa di Tropea, including the evaluation of seed production losses, were assessed. For IYSV, due to a long debated hypothesis about its presence in bulbs, experiments were carried out to determine if infected bulbs constitute an internal infection source in the field.

Materials and methods

Monitoring OYDV and IYSV incidence

This study was conducted in a municipality (Campora, CS) that is designated as the PGI area for the production of 'Rossa di Tropea'. A three-year survey was carried out in a farm where biennial cycle of onion production was followed (year 1 from seed to bulb; year 2 from bulb to seed). In particular, year 1 (bulb production) starts in September with sowing in seedbeds in an open field, followed by transplantation of seedlings in November–December and bulb harvesting in June. After three months of storage in a warehouse, bulbs are transplanted in October of year 2 (seed production) and the seeds harvested in early July (Supplementary Fig. 1).

During our survey seedbeds were in open field and no other onion crop was cultivated in close proximity, except in 2012 when a green onion crop was grown. Bulb production was accomplished in the same area of seedbeds near the sea level, whereas seed production was carried out in an area 200 m above sea level, higher than the other onion fields. Fertilization, and fungicide and pesticide treatments were as prescribed for the 'Rossa di Tropea' onion PGI production regulation. A complete biennial cycle (2012–2014) and two incomplete cycles (2012–2013 and 2013–2014) were monitored and compared to the growing stages. Methods of sampling were chosen according to plant growth stage and detection assays as described below.

Seedlings Before transplanting, more than 300 seedlings/year (15–20 cm tall) were randomly collected from seedbeds and pooled (10 seedlings per pool), then examined for virus presence by molecular assays. In cases where the pooled sample tested positive, seedlings from that pool were individually tested to determine the ratio of infection over the total number of sampled seedlings.

Dormant bulbs Bulbs were randomly collected from the storage warehouse in September 2012 and 2013. Each bulbs sample was split into two groups: part of the bulbs was used to determine the presence of OYDV and IYSV in apex tissues, and the other part was placed in pots, grown in an insect-proof greenhouse, and the emerged plants were tested for the presence of both viruses (the number of tested bulbs for each year and the virus infection are reported in Tables 2 and 4). Since IYSV was absent and OYDV-infection was very low in plants from the 2014 bulb crop, 90 bulbs collected in September 2014 were planted in a greenhouse and the resulting plants were tested only for OYDV.

Plants in the field Three plots/year of 500 plants/plot in bulb crop and 100 plants/plot in seed cultivation, respectively were randomly chosen in the field to determine the plants' phytosanitary status. A total of 1500 and 300 plants were monitored for virus symptoms twice/year, in April and in May–June, before the harvesting. During the visual monitoring of symptoms, leaf samples from symptomatic plants were collected to test virus presence by laboratory assays. In particular, leaves were collected from 10 to 30% of OYDV symptomatic plants to confirm the visual detection, whereas from 100% of suspected IYSV symptomatic plants since lesions caused by this virus could be confused with those produced by other biotic or abiotic agents.

Green onion Since a green onion cultivation was adjacent to the 2012 seedbeds, this crop was surveyed for OYDV and IYSV incidence for symptoms followed by laboratory testing of 90 plants.

Distribution of OYDV in plant and seed-transmission

The distribution of the virus was recorded in ten infected plants collected from 2013 bulb crop by testing for virus presence: roots, all bulb portions (outer tunic and inner scales, basal plate and vegetative apex) and all leaves. Furthermore, ten infected plants from 2014 seed crop were analyzed in inflorescence stems and in floral envelopes. OYDV presence was also tested in seeds by analyzing a stock of 200 seeds per year (20 samples of ten seeds each) from the 2012 and 2013 crop and 40 seeds (individually tested) from the 2014 crop. Seeds from 2012 and 2013 were provided by the farm and were collected without distinguishing if they originated from infected or healthy plants, whereas the seeds in 2014 were all collected from infected plants. Seed transmission of OYDV in 'Rossa di Tropea' onion was examined by sowing 300 seeds from each stock of 2012 and 2013, and 400 seeds of 2014 production. The experiment was carried out in an insect-proof greenhouse and seedlings were tested when were 15 cm tall. Samples were pooled in groups of five individuals.

OYDV influence on seed production

The effect of OYDV on seed production was assessed by monitoring plants ($N = 300$), from early stage to seed maturity. The assessment was done during the 2014 seed crop. Plants from this seed crop were also surveyed in July 2014. Four categories of damage were used for rating the disease severity of plants: (1) those that died soon after sprouting, (2) infected and unproductive, (3) early-infected but productive (OYDV positive in May), and (4) late-infected (OYDV positive in July). The number and the weight of seeds/inflorescence, and the weight of 100 seeds/inflorescence were determined in seeds collected from 13 plants of each productive group (3 and 4). Univariate analysis of Variance (UNIANOVA), following the general linear model (GLM) procedure, was performed with the two

productive groups (3 and 4) as fixed factors and the number of seed/inflorescence, the total weight of seed/inflorescence and the weight of 100 seeds as dependent variables. No statistical transformation was needed because the ANOVA assumptions (homogeneity and normality of variance across the groups) were not violated (Levene's and Shapiro Wilk test $p > 0.05$). Data analysis was performed by means of R version 2.3.0 (R Development Core Team 2010).

IYSV presence in bulb and bulb transmission

Part of this aim of research was studied by examination dormant bulbs randomly collected as above described (see Monitoring virus incidence: *Dormant bulb*). In addition, the migration of IYSV from infected leaves to bulbs was studied by testing the presence of IYSV in the apex of 12 fresh bulbs collected from infected but still green plants (just before harvesting time). Subsequently, to address whether the virus is transmitted from the bulb to the emerging plant, 22 IYSV-infected plants were collected from the bulb crop. Plants were left until the leaves had dried, and the bulbs were stored for three-months, then were planted in an insect-proof greenhouse. The plants from these bulbs were observed for symptoms and tested at different growing stages up to inflorescence formation.

OYDV and IYSV laboratory assays

Serological assay DAS-ELISA (Clark and Adams 1977) was used for OYDV as a detection test after visual diagnosis in the field. Commercial kits (Loewe Ghm, Sauerlach, Germany and Bioreba Reinach, Switzerland) were used following the manufacturer's instructions. ELISA reactions were measured by a spectrophotometer Multiscan FC (Thermo Scientific) at 405 nm and the samples were considered OYDV-positive when absorbance readings values were at least twice greater than the average absorbance readings of the healthy control.

Total RNA extraction Total RNA was extracted using "Real Total RNA from Tissue and Cell" kit (Durviz, Valencia, Spain) following the manufacturer's instructions. Approximately 0.5 g of plant tissue were used. The vegetative apex of the bulbs, part of seedlings after emergency, and part of the plant leaves were macerated 1:5 (w:v) in phosphate buffer. For seed assays the maceration ratio was 1 seed in 100 μL of phosphate buffer. 100 μL of the macerate were used for total RNA extraction. RNA extracted was eluted in 100 μL of nuclease-free water supplied with the kit.

Single step RT-PCR Reverse transcriptase polymerase chain reaction (RT-PCR) was used for: i) OYDV detection in dormant bulbs, seeds and seedlings with primers OYDV-NIb/CP F1 and OYDV-NIb/CP R1 that amplify a 984 bp fragment, which includes part of nuclear inclusion B (NIb) and part of coat protein (CP) of the virus (Manglli et al. 2014); and ii) IYSV detection in fresh plant material with oligonucleotides IYSV-Nc5 and IYSV/Nc3 targeting a 614 bp fragment in the nucleoprotein (N) gene (Tomassoli et al. 2009). All single-step RT-PCRs were performed in a total volume of 25 μL that consisted of 2 μL of Total RNA extract, 1X PCR buffer (Promega, Madison, WI, USA), 2.5 mM of each dNTP, 4 μM of sense and antisense primers, 1.2 U AMV-RT (Promega), 20 U RNase-OUT (Invitrogen, Carlsbad, CA, USA), 0.75 U GoTaq Polymerase (Promega), in a final volume with RNase-free water. Amplification cycles were: 46 °C for 30 min for the cDNA synthesis, followed by 5 min at 95 °C for denaturation and 35 cycles of amplification (for OYDV: 1 min at 94 °C, 1 min at 57 °C and 1 min at 72 °C; for IYSV:45 s at 94 °C, 45 s at 58 °C and 45 s at 72 °C), and a final extension at 72 °C for 10 min. RT-PCR products were visualized in 1.2% agarose gel by ethidium bromide (EtBr) staining.

Real-time RT-PCR The method was applied to determine the presence of IYSV in dormant bulbs, in plantlets generated by the bulbs, and in fresh bulbs obtained from IYSV infected plants. Oligonucleotides (IYSV 432F/503R) and probe (IYSV 455) were previously described by Tiberini et al. (2012) (Table 1). The TaqMan probe was labeled with the 6-FAM fluorophore and quencher BHQ (Applied Biosystems, Life Technologies; MGB from Sigma Company).

The reaction mixture (25 μL) contained 12.5 μL Taqman 2 X universal PCR Master Mix, 0.625 μL Multiscribe RNase Inhibitor Mix (Applied Biosystems, Life Technologies), 0.75 μL (10 μM) of each primer, 0.5 μL (5 μM) TaqMan probe, 7.875 μL RNase-free water, and 2 μL of total RNA extract. The amplification reaction was carried out in a 7500 Fast Real Time Thermocycler (Applied Biosystems) using 48 °C for 30 min of reverse-transcription, 95 °C for 10 min and 45 cycles of denaturation at 95 °C for 15 s and a step of

elongation at 60 °C for 1 min. Positive, negative and internal controls (5.8 s rRNA gene) (Robene et al. 2015), were included. Results were visualized and analyzed by SDS software (Applied Biosystems, Life Technologies). The test was duplicated for each of the samples.

Nested PCR Nested PCR was used to confirm IYSV positive results obtained in real-time RT-PCR in bulbs (dormant and fresh bulbs) and in plantlets generated from bulbs. This technique consisted of a first single-step RT-PCR reaction using the aforementioned oligonucleotides (IYSV-Nc5 / Nc3) reported above. Then, a new second set of primers was designed to target an internal 236 bp fragment of the first product obtained (Table 1).

The reaction mixture (20 μL) for nested PCR contained 1X PCR buffer (Promega, Madison, WI, USA), 2.5 mM of each dNTP, 4 μM of sense and antisense primers, 0.75 U GoTaq Polymerase (Promega), 2 μL (undiluted) of the RT-PCR product and RNase-free water. The amplification cycle was performed with an initial denaturation at 95 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min. Nested PCR amplicons were visualized in 1.2% agarose gel after EtBr staining. Data were compared with the results obtained in real-time RT-PCR and only the samples that amplified in both methods were considered positive.

Results

OYDV incidence

During the complete biennial cycle (2012–2014), at the year 1, OYDV infection in seedlings was 1.76% (6/340) which subsequently increased to 6.40% (96/1500) and reached 36.20% (543/1500) by the time the crop was ready for harvesting. Symptoms included dwarfing of plants, leaf flattening, crinkling, and yellowing (Fig. 1a). In year 2, stored bulbs had 17.86% (25/140) OYDV infection which subsequently had increased to 98.67% in the seed crop by May 2014 (Table 2). At this development stage, severe flower stem distortion and curling were observed (Fig. 1b). The green onion crop near to the seedbeds had 90% of OYDV infected plants. In year 2 of cycle 2011–2013, the initial OYDV infection of the onion seed crop was 15.87% (10/63) infected bulbs, which increased to 91.34% (274/300) of OYDV incidence by June 2013. In year 1 of the cycle 2013–2015, 0.83% (3/360) of bulb crop seedlings was infected, and in April 2014 only two symptomatic plants out of 1500 (0.13%) were found and were eradicated. In May 2014, no virus-associated symptoms were detected and testing showed only one infected sample from the surveyed plot. Moreover, bulbs collected randomly from the warehouse in September 2014 did not generate OYDV infected plants (0/90).

Distribution of OYDV in plant and seed-transmission

ELISA testing of different underground parts (root, disc as modified stem, fleshly scales as modified leaves, outer tunic and vegetative apex) of 10 plants whose leaves were found infected, did not detected the virus in roots (0/10). However, OYDV was found in the disc (9/10), in scales of bulbs (10/10) (but not in tunic), in the vegetative apex (10/10) and in each leaf. Out of 10 plants from 2014 seed crop, OYDV was found in 7 inflorescence stems, and in 10 flower envelopment.

RT-PCR testing of seeds from 2012 and 2013 (200 seeds/year, 20 samples of 10 seeds each) were all positive for OYDV. When seeds from 2014 were collected

t1.1 **Table 1** Primers and probe used in real-time RT-PCR and primer used in nested PCR for iris yellow spot tospovirus detection

Name	Sequence	Region/amplicon (bp)
Real-time RT-PCR		
IYSV 432F	5'-TTGATGCAACTACAGCCAGGAT-3′	N/72 bp
IYSV 503R	5'-AGCTGTCAAGACTCGGCAGTAAG-3′	
Probe IYSV455	5'-FAM-ATGCTGACACTGGGCGGTCCTCTC-BHQ-3′	
Nested PCR		
NC-IYSV-nestF	5'-GCTTCCTCTGGTGAGTGCAT-3'	N/236 bp
NC-IYSV-nestR	5'-CCTTTGCTGCCATGACTCTT-3'	

Fig. 1 **a**: dwarfing of onion plant with leaf flattening, crinkling and yellowing, and **b**: severe flower stem distortion and curling in onion plants caused by onion yellow dwarf virus

from infected plants and tested, 22 out of 40 were positive for OYDV. On the contrary, virus infection was not found in 599 seedlings grown from seeds in years 2012, 2013 and 2014.

OYDV influence on seed production

Among the 300 monitored plants of the 2014 seed crop, 40 plants died after sprouting and 53 did not form floriferous scape, which was equivalent to 31% loss of productive plants. All plants were infected by the seed harvesting time (July). Averages of seed number as well as the total weight of seeds produced per inflorescence obtained from 13 early-and 13 late -infected plants (categories 3 and 4 respectively) were statistically different ($F = 84.1$ and 91.5 respectively, $df = 1$, $p < 0.001$). However, averages of 100 seeds' weight of groups 3 and 4 did not differ significantly ($F = 0.243$, $df = 1$, $p = 0.626$) (Table 3).

IYSV incidence

No IYSV infection was found in 340 and 360 seedlings collected in years 2012 and 2013 respectively. The virus was detected at low percentage (1.49% and 3.57%) in bulbs randomly collected at the warehouse, however, IYSV infection in plants growing from bulbs in the greenhouse was 0%. During the three monitored years

Table 2 Incidence of onion yellow dwarf virus during three years of survey determined by RT-PCR test in seedlings and dormant bulbs after storage, and by visual inspection in the field (counting symptomatic/total plants present in selected areas), confirmed by DAS-ELISA analysis

Biennial cycles	Year 1 - Bulb production			Year 2 - Seed productions			
2011–2013	Nm			Dormant Bulbs 20-Sept-'12		Plants 16-Apr-'13	Plants 3-Jun-'13
Infected/total observed				5^a/33	5^b/30	237/300	274/300
Infection rate (%)				**15.15**	**16.67**	**79**	**91.34**
2012–2014	Seedlings 8-Nov-'12	Plants 16-Apr-'13	Plants 03-Jun-'13	11-Sept-'13		13-Apr-'14	13-May-'14
Infected/total observed	6/340	96/1500	543/1500	9^a/56	16^b/84	212/300	296/300*
Infection rate (%)	**1.76**	**6.4**	**36.2**	**16.07**	**19.04**	**70.67**	**98.67**
2013–2015	13-Dec-'13	13-Apr-'14	13-May-'14	16- Sept-'14**		Nm	Nm
Infected/total observed	3/360	2/1500	1/1500	0/90			
Infection rate (%)	**0.83**	**0.13**	**0.07**	0			

Nm: not monitored; [a] apex of dormant bulbs and [b] plants generated from dormant bulbs grown in insect-proof greenhouse were positive out of total tested by RT-PCR

*at the seed harvest time all the plants were positive for OYDV; **bulbs collected in September 2014 were all placed in pots and emerging plants were tested by RT-PCR

in both growing stages, IYSV infection did not exceeded 3.33% (Table 4) at the end of cultivation cycle. Foliar symptoms associated with IYSV consisted of small or large chlorotic and necrotic spots (Fig. 2), forming characteristic diamond-shaped lesions, single or concentric, which were evident on flower stem (Fig. 3) of seed crop. IYSV infections were detected visually and confirmed by single-step RT-PCR analysis earlier in seed than in bulb crop. In fact, 1% and 0.33% IYSV infection were detected in April 2013 and 2014 in seed crops, respectively, while in bulb crop, IYSV infection was found later in June. Moreover, the final infection rate was higher in seed crop (3.33% in 2013 and 2.67% in 2014) than in bulb crop (0.87% in 2013 and 0% in 2014). No IYSV infected plants were detected in the green onion lot near to the seedbeds during cycle 2012–2014.

IYSV presence in bulb and bulb transmission

In the context of the diagnostic assays carried out to examine the virus incidence in stored randomly collected bulbs, real-time RT-PCR and nested PCR were carried out. Real-time RT-PCR results showed the presence of IYSV in the vegetative apex of 15 out of 123 bulbs and in two out of 109 plants growing from other mother bulbs transplanted into a greenhouse. Samples yield amplification signals after 37th–38th reaction cycles, in both of the duplicated samples. Contrarily, the healthy and the majority of unknown field samples did not showed any target amplification. When nested PCR was used to test those that were positive in real-time RT-PCR, only 3/15 and 0/2 gave amplicons of expected size (Table 5). When apexes of 12 fresh bulbs of infected, but still green plants were tested, RT-PCR did not show amplification of IYSV (0/12), whereas the virus was detected both by real-time RT-PCR and by nested PCR (12/12). To determine if the virus is transmitted from bulb to the new plant, the resulting plants from other 22 bulbs from IYSV-infected plants, stored and then transplanted in the greenhouse tested all negative for IYSV in different stages of the growth. Several plants showed chlorotic/necrotic spots that were identified to be caused by *Alternaria* spp. and/or *Peronospora destructor* (, Rome, Italy).

Discussion

In 2005 outbreaks of OYDV on onion crops in Calabria was reported with rate infection up to 100% (Parrella et al. 2005). A 2012 survey on onion 'Rossa di Tropea' detected IYSV in Calabria for the first time (Manglli et al. 2012). At that time, OYDV and TSWV were also found, whereas none of the *Allexivirus, Carlavirus,* and INSV were detected (A. Manglli, personal communication), in contrast to other areas in the Mediterranean Basin (Katis et al. 2012).

In this survey OYDV was found to be most prevalent, confirming previous report from this region (Parrella et al. 2005). OYDV was detected in every stage of the complete production cycle (2012–2014), from seedlings to bulbs with 1.76%–36.2% infection rates and from stored bulbs to flowered plants with infection rates of 17.86%–98.67%. The highest infections were found in the seed crop in both of the surveyed cycles (2012–2013 and 2013–2014) starting with infection rates of 15.87% and 17.86% in stored bulbs before transplanted, and reaching the final OYDV incidence of 91.34% and 98.67%, respectively. This result showed the virus survives mainly in the bulbs, generating new infected plants which represent new sources of OYDV inoculum for the transmission by aphids. In contrast, when comparing bulb crops from cycles 2012–2013 and 2013–2014, the final OYDV incidences varied between these two cycles (36.2% and 0.07%, respectively).

It is important to highlight, that during the complete biennial cycle (2012–2014) a high infection of OYDV was detected, and a green onion crop highly infected with OYDV (about 90%) was growing near the seedbeds. It appears that similar situation of green onion crop in close proximity had existed in the previous cycle (2011–2013). It should be noted that years 2 had 91.34% OYDV infection in June. When the location of seedbeds was farther from the green onion, year 1 of last cycle (2013–2015) had 0.07% of OYDV incidence in the bulb crop and the absence of OYDV infection in stored bulbs collected in September 2014 (Table 2). Our results support that the physical isolation of seedbeds from infected crops are the most important management measures, and are in agreement with a previous study reporting a progressive declining of OYDV incidence with an increasing distance from the virus inoculum source (Ahmed and Elhassan 2013).

The importance of keeping the different production phases (seedbed, spring onion, bulb and seed

t3.1 **Table 3** Average values of number of seeds and the total weight of seeds produced per inflorescence, and average weight of 100 seeds collected from productive plants found infected by onion yellow dwarf virus in spring (early infected, group 3) and in summer (late infected, group 4). Data was analyzed by one-way variance analysis (Anova)

Infection time	No. of analyzed inflorescence	No. of seeds (mean ± SD)/ inflorescence	Total weight (gr) (mean ± SD)/ inflorescence	Weight of 100 seeds (gr) (mean ± SD)
Group 3	13	572.85 ± 49.06	2.49 ± 0.25	0.43 ± 0.03
Group 4	13	1264.23 ± 57.25	5.63 ± 0.22	0.45 ± 0.02
Sign.	–	$p < 0.001$	$p < 0.001$	Ns

Ns = not statistically significant

production) physically apart has been clearly understood by local farmers who now keep seedbeds isolated from other onion cultivations. The obtained results reinforce the importance of applying agronomical control measures during onion cultivation in order to avoid OYDV inoculum sources, and to minimize aphid-mediated virus spread (Bos 1976). However, OYDV persists in volunteer onions as reported (Schwartz and Mohan 2008). Detection of OYDV in the whole plant, with the exception of the root and the outer desiccated skins of bulbs confirmed previous reports (Sevik 2012; Velasquez-Vall et al. 2012).

The effect of OYDV infection on seed production was determined during the cycle 2013–2014. Infected bulbs caused quick decline of plants and resulted in 31% loss of productive plants. The negative effect of OYDV on seed production included reduction of weight and number seeds per inflorescence (45.31% and 44.23%, respectively), which was in agreement with previous reports (Elnagar et al. 2011; Kumar et al. 2012). However, there was no significant difference in the specific weight per seed between the two analyzed groups of plants, suggesting that there was no qualitative damage due to different times of infection. These results, even though not compared with healthy plants, were in close agreement with Rudolph (1990) who described significant decline of OYDV infection in seed yield, but not in seed quality.

It is important to underline that 599 seedlings generated from seeds collected in our survey turned out to be virus free, indicating there was no viral seed transmission in 'Rossa di Tropea'. These results are in accordance with data previously reported (Bos 1976), and differ from data by other authors (Abd El-Wahab et al. 2009) who reported OYDV transmission by seed in two Egyptian onion cvs based on OYDV detection in seed. Our results showed no transmission in grow out tests, although OYDV was detected in seed.

t4.1 **Table 4** Incidence of iris yellow spot tospovirus during three years of survey determined by real-time RT-PCR in seedlings and dormant bulbs after storage, and by visual inspections in the field (counting suspect symptomatic/total plants present in selected areas), confirmed by single step RT-PCR

Biennial cycles	Year 1 - Bulb production			Year 2 - Seed productions			
2011–2013	Nm			Dormant Bulbs 20-Sept-'12		Plants 16-Apr-'13	Plants 3-Jun-'13
Infected/total observed				1[a]/67	0[b]/69	3/300	10/300
Infection rate (%)				1.49	**0**	**1**	**3.33**
2012–2014	Seedlings 8-Nov-'12	Plants 16-Apr-13	Plants 03-Jun-'13	11-Sep-'13		13-Apr-'14	13-May-'14
Infected/total observed	0/340	0/1500	13/1500	2[a]/56	0[b]/40	1/300	8/300
Infection rate (%)	**0**	**0**	**0.87**	**3.57**	**0**	**0.33**	**2.67**
2013–2015	13-Dec-'13	13-Apr.'14	13-May-'14	Nm			
Infected/total observed	0/360	0/1500	0/1500				
Infection rate (%)	**0**	**0**	**0**				

Nm: not monitored; [a] apex of dormant bulbs and [b] plants generated from dormant bulbs grown in insect-proof greenhouse resulted positive out of total tested by real-time RT-PCR and confirmed by nested PCR

Fig. 2 Onion leaves with necrotic spots associated with infection by iris yellow spot tospovirus

Considering that in the surveyed area, onion is cultivated with a brief fallow (July–August) between two crops, and that fact that OYDV survives in infected bulbs, the relatively short allium-free time is not sufficient to break the virus reinfection pathway. Therefore, since OYDV is not transmitted by seed, the spatial separation of the seedbeds is an effective control approach for this disease in the area.

IYSV incidence was found to be low (0.87%–3.33%). In Calabria the crop cycle starts in autumn, and concludes at the beginning of summer (June) for bulb crop and in early July for seed crop. Studies conducted elsewhere showed that the incidence of symptomatic, declined plants increased after August (Hsu et al. 2010; Munoz et al. 2014) associated to the increasing of *T. tabaci* populations (Hsu et al. 2010). During this investigation, only *T. tabaci* was detected and identified at the Mediterranean University of Reggio Calabria, Italy. The virus incidence was always relatively low, maybe because of the climatic conditions of the area combined with thrips control using insecticide treatments. Further investigations are needed to better understand why IYSV spread is significantly low in this area.

IYSV-induced symptoms are easily identifiable in flower stems in seed crops allowing an reasonably accurate visual diagnosis, but less reliable on leaves of bulb crops. Severe damage caused by IYSV such as tip dieback and large necrotic spots on scapes and older leaves, resulting in reduced yield and bulb size (Nischwitz et al. 2007; Shock et al. 2008), were never observed during this investigation. Since IYSV causes considerable onion losses in Brazil, Israel and the United States (Pozzer et al. 1999; Kritzman et al. 2001; Gent et al. 2004; Pappu et al. 2009), it could represent a serious threat to the European onion production as reported by Muñoz et al. (2014).

The reappearance of IYSV, although at low levels, in each production cycle remains as an epidemiological question for further study. While IYSV is not transmitted by seed (Kritzman et al. 2001; Bulajić et al. 2009), its bulb transmission is still under discussion. In fact,

Fig. 3 Characteristic necrotic lesions, single, concentric or with diamond shape on onion flower stems caused by iris yellow spot tospovirus

t5.1 **Table 5** Results of iris yellow spot tospovirus detection in randomly collected dormant bulbs by real-time RT-PCR and nested PCR

Year of detection	2012		2013	
Diagnostic method	Apex	Generated plants	Apex	Generated plants
*Real-time RT-PCR	3/67	2/69	12/56	0/40
Nested PCR	**1/3	**0/2	**2/12	Nm

*Real-time RT-PCR showed amplification reactions at late cycles (>37, 38 Ct), **Samples were considered positive when confirmed by nested PCR. Nm: not monitored

Kritzman et al. (2001) did not identify bulbs as virus inoculum sources, whereas other authors reported infection in bulbs (Robène-Soustrade et al. 2006; Weilner and Bedlan 2013). Schwartz (2008) did not exclude that IYSV can be transmitted by bulbs despite later the virus was not detected in bulb scales and basal plates by ELISA (Boateng and Schwartz 2013). A real-time RT-PCR test was shown to be more sensitive than the previously used methods, allowing the detection of IYSV in apex, but not in other bulb portions (Tiberini et al. 2011).

This study used real-time RT-PCR and nested PCR to test the bulb vegetative internal apex. It appears that IYSV migrates into vegetative apex, but it becomes quiescent/inactive in stored bulbs. Moreover, a relatively low viral concentration inside the bulb (detectable only by highly sensitive diagnostic methods), may not be sufficient for the establishment of infection and disease development in the newly generated plants in our experimental conditions.

However, in this study IYSV was always detected first in seed crops than in bulb crops, and the final infection rate was always higher in seed than in bulb crops, suggesting there was an internal source of viral inoculum in the field. Schwartz (2008) reported a positive IYSV transmission from bulbs to the newly generated plants even though the authors could not attribute the source of infection to virus presence or viruliferous thrips in bulbs. According to this previous report and our results the epidemiology of bulb transmission deserves further attention. In particular, a study aimed at examining the thrips' life cycle and the presence of viruliferous individuals during bulb storage, and their role in IYSV infection during the seed production cycle of onion, all of which will provide new important insights into IYSV epidemiology.

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Compliance with ethical standards

Ethical statement This research did not involve any animal and/or human participant. The authors declare that they have no conflict of interest.

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