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67	Abstract	Accepted 25 December 2019 A survey on the incidence of onion yellow dwarf virus (OYDV) and iris yellow to spovirus (IYSV) was carried out over three production cycles of one '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 seedlings with 1.76% infection rate determining 36.2% and 98.67% infect plants in the bulbs and in the subsequent seed harvesting times, respective When seedbeds were at least one km away from other onion crops seedling and bulb cultivation had the infection rate close to zero. OYDV was detect 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 compare late season infection, while the weight of 100 seeds was not different in the 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), at 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 field. IYSV was detected in 3/123 apex bulbs randomly collected from sto 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 contrar randomly collected bulbs ($N = 109$) from warehouse and bulbs of 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

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17Abstract A survey on the incidence of onion yellow dwarf virus (OYDV) and iris yellow spot tospovirus 18(IYSV) was carried out over three production cycles of 1920onion 'Rossa di Tropea' in Calabria, Italy. OYDV was found to be the prevalent virus. 'Rossa di Tropea' seed 2122adjacent to OYDV-infected green onion field had seed-23lings with 1.76% infection rate determining 36.2% and 98.67% infected plants in the bulbs and in the subse-24quent seed harvesting times, respectively. When seed-2526beds were at least one km away from other onion crops seedlings and bulb cultivation had the infection rate 27close to zero. OYDV was detected in whole plants 2829except the roots and outer desiccated bulb skins. Seed transmission was not detected in 'Rossa di Tropea'. 30 31 Early OYDV infection significantly reduced the number 32 and weight of seeds/inflorescence compared to late

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season infection, while the weight of 100 seeds was 33 not different in the two early and late OYDV infected 34plants. IYSV was never found in seedbeds. It was al-35ways detected first in seed crops (April) than in bulb 36 crops (June), and the final infection rate was higher in 37 seed (2.67%–3.33%) than in bulb crops (0%–0.87%), 38suggesting there was an internal source of viral inocu-39lum in the field. IYSV was detected in 3/123 apex bulbs 40randomly collected from stored bulbs and in 12/12 apex 41 fresh bulbs collected at harvest time from infected 42plants, showing the role of bulbs as IYSV inoculum 43source. On the contrary, randomly collected bulbs 44 (N=109) from warehouse and bulbs of infected plants 45(N=22), transplanted after storage, did not result in 46IYSV-infected plants. 47

KeywordsOnion cv Rossa di Tropea · OYDV · IYSV ·48Italy · Virus detection · Virus transmission49

Introduction

Onion (Allium cepa L.) is the most widely cultivated 51species of the genus Allium, family Amaryllidaceae. 52The production of onion in Italy was of 410,535 tons 53in 2017 on 12,248 ha (ISTAT 2017) including many 54 02 cultivars/biotypes of high organoleptic quality. In Cala-55bria, southern Italy, onion production was about 34,700 56tons cultivated on 922 ha (ISTAT 2017). In this region, 57'Rossa di Tropea' onion holds a Protected Geographical 58Indication (PGI) trademark. Soil and climatic character-59istics for the cultivation of 'Rossa di Tropea' extend 60

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, 65 and viruses affect onion. The viral species known to 66 infect onion belong to the genera Allexivirus, 67 Carlavirus, Potyvirus, and Orthotospovirus. Among 68 them, potyviruses and orthotospoviruses induce the 69 most important damage to this crop, whereas 70allexiviruses and carlaviruses cause latent infections, 71but cause perceptible damage in mixed infection with 72potyviruses (Katis et al. 2012). 73

Amongst members of the genus Potyvirus, the spe-74cies Onion yellow dwarf virus (OYDV) is the most 75economically important virus affecting Allium species 76(Van Dijk 1993), especially onion and garlic. OYDV is 77transmitted in a non persistent manner by more than 50 78aphid species (Drake et al. 1933), and Myzus persicae 79(Sulzer) is reported as the most efficient vector, followed 80 81 by Aphis craccivora (Koch) and A. gossypii (Glover) 82 (Abd El-Wahab 2009; Kumar et al. 2011). OYDV has a host range limited to Allium species and is detectable in 83 almost all onion and garlic growing regions of the world 84 85 (Dovas et al. 2001; Pappu et al. 2005; Katis et al. 2012; Chodorska et al. 2014; Majumder and Johari 2014; 86 Vončina et al. 2016; Majumder et al. 2017; Sivaprasad 87 et al. 2017). In Italy, OYDV was first reported based on 88 symptoms on onions (Marani and Bertaccini 1983); the 89 virus was later confirmed by molecular assays (Dovas 90 and Volvas 2003), and 100% of infection in 'Rossa di 91Tropea' onion was reported in Calabria (Parrella et al. 92 2005). OYDV causes severe economic losses both in 93 onion seed and bulb crops (Hoa et al. 2003; Elnagar 94 et al. 2011; Kumar et al. 2012). The virus remains 95persistent in onion sprouts, plant residues and bulbs 96 (Schwartz and Mohan 2008; Katis et al. 2012) whereas 97 seed transmission was reported only in a few local 98 99cultivars (Ibrahim et al. 1996; Abd El-Wahab et al. 2009). 100

101 Three species of orthotospoviruses are able to naturally infect onion: Impatiens necrotic spot tospovirus 102(INSV), Iris vellow spot tospovirus (IYSV), and Tomato 103104 spotted wilt tospovirus (TSWV) (Mullis et al. 2004; Vicchi et al. 2008; Pappu et al. 2009). IYSV was first 105identified in Iris hollandica (Cortês et al. 1998). IYSV 106107 became an important pathogen that causes severe disease in onion worldwide, especially in North and South 108 America (Pozzer et al. 1999; Crowe and Pappu 2005; 109

Gent et al. 2006; Hoepting et al. 2007, 2008; Bag et al. 1102015). The virus is transmitted by Thrips tabaci 111Lindeman (Cortês et al. 1998; Nagata et al. 1999; 112Krizman et al. 2001) and less efficiently by 11303 Frankliniella fusca Hinds (Srinivasan et al. 2012). In 114the Mediterranean basin, IYSV was reported in Spain 115(Cordoba-Selles et al. 2005; Muñoz et al. 2014), France 116(Huchette et al. 2008), and Italy (Tomassoli et al. 2009; 117Manglli et al. 2012; Turina et al. 2012). The virus is not 118seed-transmitted (Krizman et al. 2001; Bulajić et al. 1192009), but bulb transmission was suggested (Robène-120 Soustrade et al. 2006; Schwartz 2008; Weilner and 121Bedlan 2013), although the presence of the virus in 122bulbs has not been reported by other authors 123(Kritzman et al. 2001; Boateng and Schwartz 2013). 124

In this study, the incidence of OYDV and IYSV was 125investigated during three production cycles (2012-1262015) in Calabria aiming to develop effective manage-127ment practices to ensure better yields of 'Rossa di 128Tropea'. Due to the different features of these two 129viruses, two parallel and independent approaches were 130 used, starting from surveys to assess the incidence of 131OYDV and IYSV in different production phases by 132visual inspection and laboratory assays. Moreover, for 133OYDV, some disease aspects such as virus presence in 134various plant parts, and virus transmission through seed 135in cv. Rossa di Tropea, including the evaluation of seed 136production losses, were assessed. For IYSV, due to a 137 long debated hypothesis about its presence in bulbs, 138experiments were carried out to determine if infected 139bulbs constitute an internal infection source in the field. 140

Materials and methods 141

Monitoring OYDV and IYSV incidence	142
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This study was conducted in a municipality (Campora, 143 CS) that is designated as the PGI area for the production 144 of 'Rossa di Tropea'. A three-year survey was carried 145out in a farm where biennial cycle of onion production 146was followed (year 1 from seed to bulb; year 2 from 147bulb to seed). In particular, year 1 (bulb production) 148starts in September with sowing in seedbeds in an open 149field, followed by transplantation of seedlings in 150November-December and bulb harvesting in June. Af-151ter three months of storage in a warehouse, bulbs are 152transplanted in October of year 2 (seed production) and 153the seeds harvested in early July (Supplementary Fig. 1). 154

During our survey seedbeds were in open field and no 155other onion crop was cultivated in close proximity, 156except in 2012 when a green onion crop was grown. 157Bulb production was accomplished in the same area of 158seedbeds near the sea level, whereas seed production 159was carried out in an area 200 m above sea level, higher 160 than the other onion fields. Fertilization, and fungicide 161and pesticide treatments were as prescribed for the 162'Rossa di Tropea' onion PGI production regulation. A 163164 complete biennial cycle (2012-2014) and two incomplete cycles (2012-2013 and 2013-2014) were moni-165tored and compared to the growing stages. Methods of 166167 sampling were chosen according to plant growth stage and detection assays as described below. 168

169 Seedlings Before transplanting, more than 300 seedlings/year (15-20 cm tall) were randomly collected 170from seedbeds and pooled (10 seedlings per pool), then 171examined for virus presence by molecular assays. In 172cases where the pooled sample tested positive, seedlings 173from that pool were individually tested to determine the 174175ratio of infection over the total number of sampled seedlings. 176

177Dormant bulbs Bulbs were randomly collected from the storage warehouse in September 2012 and 2013. Each 178bulbs sample was split into two groups: part of the bulbs 179was used to determine the presence of OYDV and IYSV 180 in apex tissues, and the other part was placed in pots, 181 grown in an insect-proof greenhouse, and the emerged 182plants were tested for the presence of both viruses (the 183 number of tested bulbs for each year and the virus 184infection are reported in Tables 2 and 4). Since IYSV 185was absent and OYDV-infection was very low in plants 186from the 2014 bulb crop, 90 bulbs collected in Septem-187 ber 2014 were planted in a greenhouse and the resulting 188 plants were tested only for OYDV. 189

190Plants in the field Three plots/year of 500 plants/plot in bulb crop and 100 plants/plot in seed cultivation, re-191192spectively were randomly chosen in the field to determine the plants' phytosanitary status. A total of 1500 193and 300 plants were monitored for virus symptoms 194 195twice/year, in April and in May-June, before the harvesting. During the visual monitoring of symptoms, leaf 196 samples from symptomatic plants were collected to test 197 198 virus presence by laboratory assays. In particular, leaves were collected from 10 to 30% of OYDV symptomatic 199plants to confirm the visual detection, whereas from 200

100% of suspected IYSV symptomatic plants since201lesions caused by this virus could be confused with202those produced by other biotic or abiotic agents.203

Green onion Since a green onion cultivation was adja-
cent to the 2012 seedbeds, this crop was surveyed for
OYDV and IYSV incidence for symptoms followed by
laboratory testing of 90 plants.204
205

Distribution of OYDV in plant and seed-transmission 208

The distribution of the virus was recorded in ten infected 209plants collected from 2013 bulb crop by testing for virus 210 presence: roots, all bulb portions (outer tunic and inner 211scales, basal plate and vegetative apex) and all leaves. 212Furthermore, ten infected plants from 2014 seed crop 213were analyzed in inflorescence stems and in floral en-214velopes. OYDV presence was also tested in seeds by 215analyzing a stock of 200 seeds per year (20 samples of 216ten seeds each) from the 2012 and 2013 crop and 40 217seeds (individually tested) from the 2014 crop. Seeds 218from 2012 and 2013 were provided by the farm and 219were collected without distinguishing if they originated 220 from infected or healthy plants, whereas the seeds in 2212014 were all collected from infected plants. Seed trans-222mission of OYDV in 'Rossa di Tropea' onion was 223examined by sowing 300 seeds from each stock of 2242012 and 2013, and 400 seeds of 2014 production. 225The experiment was carried out in an insect-proof green-226 house and seedlings were tested when were 15 cm tall. 227Samples were pooled in groups of five individuals. 228

OYDV influence on seed production

The effect of OYDV on seed production was assessed by 230monitoring plants (N = 300), from early stage to seed 231maturity. The assessment was done during the 2014 seed 232crop. Plants from this seed crop were also surveyed in 233July 2014. Four categories of damage were used for 234rating the disease severity of plants: (1) those that died 235soon after sprouting, (2) infected and unproductive, (3) 236early-infected but productive (OYDV positive in May), 237and (4) late-infected (OYDV positive in July). The 238number and the weight of seeds/inflorescence, and the 239weight of 100 seeds/inflorescence were determined in 240seeds collected from 13 plants of each productive group 241(3 and 4). Univariate analysis of Variance 242(UNIANOVA), following the general linear model 243(GLM) procedure, was performed with the two 244

productive groups (3 and 4) as fixed factors and the 245number of seed/inflorescence, the total weight of 246seed/inflorescence and the weight of 100 seeds as de-247pendent variables. No statistical transformation was 248needed because the ANOVA assumptions (homogeneity 249and normality of variance across the groups) were not 250violated (Levene's and Shapiro Wilk test p > 0.05). Data 251analysis was performed by means of R version 2.3.0 (R 252

253 Development Core Team 2010).

254 IYSV presence in bulb and bulb transmission

255Part of this aim of research was studied by examination dormant bulbs randomly collected as above described 256(see Monitoring virus incidence: Dormant bulb). In 257addition, the migration of IYSV from infected leaves 258to bulbs was studied by testing the presence of IYSV in 259the apex of 12 fresh bulbs collected from infected but 260261still green plants (just before harvesting time). Subsequently, to address whether the virus is transmitted from 262the bulb to the emerging plant, 22 IYSV-infected plants 263264were collected from the bulb crop. Plants were left until the leaves had dried, and the bulbs were stored for three-265months, then were planted in an insect-proof green-266267house. The plants from these bulbs were observed for symptoms and tested at different growing stages up to 268inflorescence formation. 269

270 OYDV and IYSV laboratory assays

271Serological assay DAS-ELISA (Clark and Adams 2721977) was used for OYDV as a detection test after visual diagnosis in the field. Commercial kits (Loewe Ghm, 273Sauerlach, Germany and Bioreba Reinach, Switzerland) 274were used following the manufacturer's instructions. 275276ELISA reactions were measured by a spectrophotometer Multiscan FC (Thermo Scientific) at 405 nm and the 277278samples were considered OYDV-positive when absor-279bance readings values were at least twice greater than the average absorbance readings of the healthy control. 280

Total RNA extraction Total RNA was extracted using 281282 "Real Total RNA from Tissue and Cell" kit (Durviz, 283Valencia, Spain) following the manufacturer's instructions. Approximately 0.5 g of plant tissue were used. 284The vegetative apex of the bulbs, part of seedlings after 285286 emergency, and part of the plant leaves were macerated 1:5 (w:v) in phosphate buffer. For seed assays the mac-287eration ratio was 1 seed in 100 µL of phosphate buffer. 288

 $\begin{array}{ll} 100 \ \mu L \ of \ the \ macerate \ were \ used \ for \ total \ RNA \ extrac- \\ tion. \ RNA \ extracted \ was \ eluted \ in \ 100 \ \mu L \ of \ nuclease- \\ free \ water \ supplied \ with \ the \ kit. \\ \end{array} \begin{array}{ll} 290 \\ 291 \end{array}$

Single step RT-PCR Reverse transcriptase polymerase 292chain reaction (RT-PCR) was used for: i) OYDV detec-293tion in dormant bulbs, seeds and seedlings with primers 294OYDV-NIb/CP F1 and OYDV-NIb/CP R1 that amplify 295a 984 bp fragment, which includes part of nuclear in-296clusion B (NIb) and part of coat protein (CP) of the virus 297(Manglli et al. 2014); and ii) IYSV detection in fresh 298 plant material with oligonucleotides IYSV-Nc5 and 299IYSV/Nc3 targeting a 614 bp fragment in the nucleo-300protein (N) gene (Tomassoli et al. 2009). All single-step 301RT-PCRs were performed in a total volume of 25 µL 302 that consisted of 2 µL of Total RNA extract, 1X PCR 303 buffer (Promega, Madison, WI, USA), 2.5 mM of each 304 dNTP, 4 µM of sense and antisense primers, 1.2 U 305 AMV-RT (Promega), 20 U RNase-OUT (Invitrogen, 306 Carlsbad, CA, USA), 0.75 U GoTaq Polymerase 307 (Promega), in a final volume with RNase-free water. 308 Amplification cycles were: 46 °C for 30 min for the 309 cDNA synthesis, followed by 5 min at 95 °C for dena-310 turation and 35 cycles of amplification (for OYDV: 3111 min at 94 °C, 1 min at 57 °C and 1 min at 72 °C; for 312IYSV:45 s at 94 °C, 45 s at 58 °C and 45 s at 72 °C), and 313a final extension at 72 °C for 10 min. RT-PCR products 314were visualized in 1.2% agarose gel by ethidium bro-315mide (EtBr) staining. 316

Real-time RT-PCR The method was applied to deter-317mine the presence of IYSV in dormant bulbs, in plantlets 318generated by the bulbs, and in fresh bulbs obtained from 319IYSV infected plants. Oligonucleotides (IYSV 320432F/503R) and probe (IYSV 455) were previously 321described by Tiberini et al. (2012) (Table 1). The 322 TaqMan probe was labeled with the 6-FAM fluorophore 323and quencher BHQ (Applied Biosystems, Life Technol-324 ogies; MGB from Sigma Company). 325

The reaction mixture (25 μ L) contained 12.5 μ L 326 Taqman 2 X universal PCR Master Mix, 0.625 µL 327 Multiscribe RNase Inhibitor Mix (Applied Biosystems, 328 Life Technologies), 0.75 µL (10 µM) of each primer, 329 0.5 µL (5 µM) TaqMan probe, 7.875 µL RNase-free 330 water, and 2 µL of total RNA extract. The amplification 331reaction was carried out in a 7500 Fast Real Time 332Thermocycler (Applied Biosystems) using 48 °C for 33330 min of reverse-transcription, 95 °C for 10 min and 334 45 cycles of denaturation at 95 °C for 15 s and a step of 335 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.

342 Nested PCR Nested PCR was used to confirm IYSV positive results obtained in real-time RT-PCR in bulbs 343(dormant and fresh bulbs) and in plantlets generated from 344bulbs. This technique consisted of a first single-step RT-345PCR reaction using the aforementioned oligonucleotides 346347 (IYSV-Nc5 / Nc3) reported above. Then, a new second set of primers was designed to target an internal 236 bp 348fragment of the first product obtained (Table 1). 349

350The reaction mixture (20 µL) for nested PCR contained 1X PCR buffer (Promega, Madison, WI, 351USA), 2.5 mM of each dNTP, 4 µM of sense and 352353antisense primers, 0.75 U GoTaq Polymerase (Promega), 2 µL (undiluted) of the RT-PCR product 354and RNase-free water. The amplification cycle was per-355356 formed 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, 357 72 °C for 1 min, and a final extension at 72 °C for 5 min. 358 359 Nested PCR amplicons were visualized in 1.2% agarose gel after EtBr staining. Data were compared with the 360 results obtained in real-time RT-PCR and only the sam-361ples that amplified in both methods were considered 362 positive. 363

364 Results

365 OYDV incidence

366 During the complete biennial cycle (2012–2014), at the 367 year 1, OYDV infection in seedlings was 1.76% (6/340) which subsequently increased to 6.40% (96/1500) and 368 reached 36.20% (543/1500) by the time the crop was 369 ready for harvesting. Symptoms included dwarfing of 370plants, leaf flattening, crinkling, and yellowing (Fig. 1a). 371In year 2, stored bulbs had 17.86% (25/140) OYDV 372infection which subsequently had increased to 98.67% 373 in the seed crop by May 2014 (Table 2). At this devel-374opment stage, severe flower stem distortion and curling 375 were observed (Fig. 1b). The green onion crop near to 376 the seedbeds had 90% of OYDV infected plants. In year 377 2 of cycle 2011–2013, the initial OYDV infection of the 378 onion seed crop was 15.87% (10/63) infected bulbs, 379 which increased to 91.34% (274/300) of OYDV inci-380 dence by June 2013. In year 1 of the cycle 2013–2015, 3810.83% (3/360) of bulb crop seedlings was infected, and 382 in April 2014 only two symptomatic plants out of 1500 383 (0.13%) were found and were eradicated. In May 2014, 384 no virus-associated symptoms were detected and testing 385showed only one infected sample from the surveyed 386plot. Moreover, bulbs collected randomly from the 387 warehouse in September 2014 did not generate OYDV 388 infected plants (0/90). 389

Distribution of OYDV in plant and seed-transmission 390

ELISA testing of different underground parts (root, disc 391as modified stem, fleshly scales as modified leaves, 392 outer tunic and vegetative apex) of 10 plants whose 393 leaves were found infected, did not detected the virus 394 in roots (0/10). However, OYDV was found in the disc 395(9/10), in scales of bulbs (10/10) (but not in tunic), in the 396 vegetative apex (10/10) and in each leaf. Out of 10 397 plants from 2014 seed crop, OYDV was found in 7 398 inflorescence stems, and in 10 flower envelopment. 399

RT-PCR testing of seeds from 2012 and 2013 (200400seeds/year, 20 samples of 10 seeds each) were all pos-401itive for OYDV. When seeds from 2014 were collected402

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 IYSV 503R	5'-TTGATGCAACTACAGCCAGGAT-3' 5'-AGCTGTCAAGACTCGGCAGTAAG-3'	N/72 bp
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

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- 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.
- 407 OYDV influence on seed production

408 Among the 300 monitored plants of the 2014 seed crop,
409 40 plants died after sprouting and 53 did not form
410 floriferous scape, which was equivalent to 31% loss of

411 productive plants. All plants were infected by the seed

412 harvesting time (July). Averages of seed number as well

as the total weight of seeds produced per inflorescence

414 obtained from 13 early-and 13 late -infected plants

Q4t2.1

 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
 IYSV incidence

0.626) (Table 3).

No IYSV infection was found in 340 and 360 seedlings421collected in years 2012 and 2013 respectively. The virus422was detected at low percentage (1.49% and 3.57%) in423bulbs randomly collected at the warehouse, however,424IYSV infection in plants growing from bulbs in the425greenhouse was 0%. During the three monitored years426

(categories 3 and 4 respectively) were statistically dif-

ferent (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 =

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 20-Sept-	Bulbs 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	343/1500	9750	10 /84	212/300	296/300*	
2013–2015	1.70 13-Dec-'13	0.4 13-Apr-'14	30.2 13-May-'14	16- Sept-	19.04 -'14**	7 0.6 7 Nm	98.07 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 427 3.33% (Table 4) at the end of cultivation cycle. Foliar 428 symptoms associated with IYSV consisted of small or 429large chlorotic and necrotic spots (Fig. 2), forming char-430acteristic diamond-shaped lesions, single or concentric, 431which were evident on flower stem (Fig. 3) of seed crop. 432IYSV infections were detected visually and confirmed 433by single-step RT-PCR analysis earlier in seed than in 434 bulb crop. In fact, 1% and 0.33% IYSV infection were 435detected in April 2013 and 2014 in seed crops, respec-436tively, while in bulb crop, IYSV infection was found 437 later in June. Moreover, the final infection rate was 438439 higher in seed crop (3.33% in 2013 and 2.67% in 2014) than in bulb crop (0.87% in 2013 and 0% in 440 2014). No IYSV infected plants were detected in the 441 442 green onion lot near to the seedbeds during cycle 2012-2014. 443

444 IYSV presence in bulb and bulb transmission

445In the context of the diagnostic assays carried out to examine the virus incidence in stored randomly 446 collected bulbs, real-time RT-PCR and nested PCR 447 were carried out. Real-time RT-PCR results 448 showed the presence of IYSV in the vegetative 449apex of 15 out of 123 bulbs and in two out of 450109 plants growing from other mother bulbs 451 transplanted into a greenhouse. Samples yield am-452plification signals after 37th-38th reaction cycles, 453in both of the duplicated samples. Contrarily, the 454healthy and the majority of unknown field samples 455did not showed any target amplification. When 456nested PCR was used to test those that were 457positive in real-time RT-PCR, only 3/15 and 0/2 458gave amplicons of expected size (Table 5). When 459apexes of 12 fresh bulbs of infected, but still 460green plants were tested, RT-PCR did not show 461462amplification of IYSV (0/12), whereas the virus was detected both by real-time RT-PCR and by 463 464nested PCR (12/12). To determine if the virus is transmitted from bulb to the new plant, the 465resulting plants from other 22 bulbs from IYSV-466 467 infected plants, stored and then transplanted in the greenhouse tested all negative for IYSV in differ-468 ent stages of the growth. Several plants showed 469 470 chlorotic/necrotic spots that were identified to be caused by Alternaria spp. and/or Peronospora 471472 destructor (, Rome, Italy).

Discussion

In 2005 outbreaks of OYDV on onion crops in Calabria 474was reported with rate infection up to 100% (Parrella 475et al. 2005). A 2012 survey on onion 'Rossa di Tropea' 476 detected IYSV in Calabria for the first time (Manglli 477 et al. 2012). At that time, OYDV and TSWV were also 478found, whereas none of the Allexivirus, Carlavirus, and 479 INSV were detected (A. Manglli, personal communica-480 tion), in contrast to other areas in the Mediterranean 481Basin (Katis et al. 2012). 482

In this survey OYDV was found to be most preva-483 lent, confirming previous report from this region 484(Parrella et al. 2005). OYDV was detected in every stage 485of the complete production cycle (2012-2014), from 486 seedlings to bulbs with 1.76%-36.2% infection rates 487 and from stored bulbs to flowered plants with infection 488 rates of 17.86%-98.67%. The highest infections were 489found in the seed crop in both of the surveyed cycles 490(2012-2013 and 2013-2014) starting with infection 491rates of 15.87% and 17.86% in stored bulbs before 492transplanted, and reaching the final OYDV incidence 493of 91.34% and 98.67%, respectively. This result showed 494 the virus survives mainly in the bulbs, generating new 495infected plants which represent new sources of OYDV 496 inoculum for the transmission by aphids. In contrast, 497 when comparing bulb crops from cycles 2012-2013 and 498 2013-2014, the final OYDV incidences varied between 499these two cycles (36.2% and 0.07%, respectively). 500

It is important to highlight, that during the complete 501biennial cycle (2012-2014) a high infection of OYDV 502was detected, and a green onion crop highly infected 503with OYDV (about 90%) was growing near the seed-504beds. It appears that similar situation of green onion crop 505in close proximity had existed in the previous cycle 506(2011-2013). It should be noted that years 2 had 50791.34% OYDV infection in June. When the location of 508seedbeds was farther from the green onion, year 1 of last 509cycle (2013-2015) had 0.07% of OYDV incidence in 510the bulb crop and the absence of OYDV infection in 511stored bulbs collected in September 2014 (Table 2). Our 512results support that the physical isolation of seedbeds 513from infected crops are the most important management 514measures, and are in agreement with a previous study 515reporting a progressive declining of OYDV incidence 516with an increasing distance from the virus inoculum 517source (Ahmed and Elhassan 2013). 518

The importance of keeping the different production 519 phases (seedbed, spring onion, bulb and seed 520

seeds cone	celea nom productive	plants found infected by onion	variance analysis (/ mova)	
Infection time	No. of analyzed inflorescence	No. of seeds (mean \pm SD)/ inflorescence	Total weight (gr) (mean \pm 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

 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)

Ns = not statistically significant

t3.1

production) physically apart has been clearly under-521522stood by local farmers who now keep seedbeds isolated from other onion cultivations. The obtained results re-523inforce the importance of applying agronomical control 524measures during onion cultivation in order to avoid 525OYDV inoculum sources, and to minimize aphid-526 mediated virus spread (Bos 1976). However, OYDV 527persists in volunteer onions as reported (Schwartz and 528Mohan 2008). Detection of OYDV in the whole plant, 529with the exception of the root and the outer desiccated 530skins of bulbs confirmed previous reports (Sevik 2012; 531Velasquez-Vall et al. 2012). 532

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). How-540ever, there was no significant difference in the specific 541weight per seed between the two analyzed groups of 542plants, suggesting that there was no qualitative damage 543due to different times of infection. These results, even 544though not compared with healthy plants, were in close 545agreement with Rudolph (1990) who described signifi-546cant decline of OYDV infection in seed yield, but not in 547seed quality. 548

It is important to underline that 599 seedlings gener-549ated from seeds collected in our survey turned out to be 550virus free, indicating there was no viral seed transmis-551sion in 'Rossa di Tropea'. These results are in accor-552dance with data previously reported (Bos 1976), and 553differ from data by other authors (Abd El-Wahab et al. 5542009) who reported OYDV transmission by seed in two 555Egyptian onion cvs based on OYDV detection in seed. 556Our results showed no transmission in grow out tests, 557although OYDV was detected in seed. 558

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	Biennial cycles Year 1 - Bulb production			Year 2 -	Year 2 - Seed productions				
2011–2013	Nm			Dormant 20-Sept-	Bulbs 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 Infected/total observed	13-Dec-'13 0/360	13-Apr.'14 0/1500	13-May-'14 0/1500	Nm					
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

559Considering that in the surveyed area, onion is cultivated with a brief fallow (July-August) between two 560crops, and that fact that OYDV survives in infected 561562bulbs, the relatively short allium-free time is not sufficient to break the virus reinfection pathway. Therefore, 563since OYDV is not transmitted by seed, the spatial 564 565 separation of the seedbeds is an effective control approach for this disease in the area. 566

IYSV incidence was found to be low (0.87%-5675683.33%). In Calabria the crop cycle starts in autumn, and concludes at the beginning of summer (June) for 569bulb crop and in early July for seed crop. Studies con-570571ducted elsewhere showed that the incidence of symptomatic, declined plants increased after August (Hsu 572et al. 2010; Munoz et al. 2014) associated to the increas-573574ing of T. tabaci populations (Hsu et al. 2010). During this investigation, only T. tabaci was detected and iden-575tified at the Mediterranean University of Reggio Cala-576577 bria, Italy. The virus incidence was always relatively low, maybe because of the climatic conditions of the 578579 area combined with thrips control using insecticide

treatments. Further investigations are needed to better 580 understand why IYSV spread is significantly low in this 581 area. 582

IYSV-induced symptoms are easily identifiable in 583flower stems in seed crops allowing an reasonably ac-584curate visual diagnosis, but less reliable on leaves of 585bulb crops. Severe damage caused by IYSV such as tip 586dieback and large necrotic spots on scapes and older 587 leaves, resulting in reduced yield and bulb size 588(Nischwitz et al. 2007; Shock et al. 2008), were never 589observed during this investigation. Since IYSV causes 590considerable onion losses in Brazil, Israel and the Unit-591ed States (Pozzer et al. 1999; Kritzman et al. 2001; Gent 592et al. 2004; Pappu et al. 2009), it could represent a 593serious threat to the European onion production as re-594ported by Muñoz et al. (2014). 595

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, 600

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	vellow spot tos	povirus detection i	in randomly collecte	ed dormant bulbs l	by real-time RT-PCR and nested PCR
				2		

	2012		2012				
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

601 Kritzman et al. (2001) did not identify bulbs as virus inoculum sources, whereas other authors reported infec-602 tion in bulbs (Robène-Soustrade et al. 2006; Weilner 603 and Bedlan 2013). Schwartz (2008) did not exclude that 604 IYSV can be transmitted by bulbs despite later the virus 605 was not detected in bulb scales and basal plates by 606 607 ELISA (Boateng and Schwartz 2013). A real-time RT-PCR test was shown to be more sensitive than the 608 previously used methods, allowing the detection of 609 IYSV in apex, but not in other bulb portions (Tiberini 610 et al. 2011). 611

This study used real-time RT-PCR and nested PCR to 612 test the bulb vegetative internal apex. It appears that 613 IYSV migrates into vegetative apex, but it becomes 614 quiescent/inactive in stored bulbs. Moreover, a relative-615 616 ly low viral concentration inside the bulb (detectable only by highly sensitive diagnostic methods), may not 617 be sufficient for the establishment of infection and 618 619 disease development in the newly generated plants in our experimental conditions. 620

However, in this study IYSV was always detected 621 622 first in seed crops than in bulb crops, and the final infection rate was always higher in seed than in bulb 623 crops, suggesting there was an internal source of viral 624 625 inoculum in the field. Schwartz (2008) reported a positive IYSV transmission from bulbs to the newly gener-626 ated plants even though the authors could not attribute 627 628 the source of infection to virus presence or viruliferous thrips in bulbs. According to this previous report and our 629 results the epidemiology of bulb transmission deserves 630 631 further attention. In particular, a study aimed at examining the thrips' life cycle and the presence of viruliferous 632 individuals during bulb storage, and their role in IYSV 633 634 infection during the seed production cycle of onion, all of which will provide new important insights into IYSV 635 epidemiology. 636

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Compliance	with	ethical	standards
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Ethical statementThis research did not involve any animal and/654or human participant. The authors declare that they have no655conflict of interest.656

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