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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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## Electronic supplementary material

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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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**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

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**Keywords** Onion cv Rossa di Tropea · OYDV · IYSV · Italy · Virus detection · Virus transmission

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## 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

61 through the provinces of Catanzaro, Cosenza, and Vibo  
62 Valentia, and confer to this cultivar its distinctive organo-  
63 leptic properties that are highly appreciated in Italy and  
64 abroad where exported.

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

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

101 Three species of orthospoviruses are able to natu-  
102 rally infect onion: *Impatiens necrotic spot tospovirus*  
103 (INSV), *Iris yellow spot tospovirus* (IYSV), and *Tomato*  
104 *spotted wilt tospovirus* (TSWV) (Mullis et al. 2004;  
105 Vicchi et al. 2008; Pappu et al. 2009). IYSV was first  
106 identified in *Iris hollandica* (Cortês et al. 1998). IYSV  
107 became an important pathogen that causes severe dis-  
108 ease in onion worldwide, especially in North and South  
109 America (Poizzer et al. 1999; Crowe and Pappu 2005;

Gent et al. 2006; Hoepting et al. 2007, 2008; Bag et al. 110  
2015). The virus is transmitted by *Thrips tabaci* 111  
Lindeman (Cortês et al. 1998; Nagata et al. 1999; 112  
Krizman et al. 2001) and less efficiently by 113Q3  
*Frankliniella fusca* Hinds (Srinivasan et al. 2012). In 114  
the 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; 117  
Manglli et al. 2012; Turina et al. 2012). The virus is not 118  
seed-transmitted (Krizman et al. 2001; Bulajić et al. 119  
2009), but bulb transmission was suggested (Robène- 120  
Soustrade et al. 2006; Schwartz 2008; Weilner and 121  
Bedlan 2013), although the presence of the virus in 122  
bulbs has not been reported by other authors 123  
(Kritzman et al. 2001; Boateng and Schwartz 2013). 124

125 In this study, the incidence of OYDV and IYSV was 125  
investigated during three production cycles (2012– 126  
2015) in Calabria aiming to develop effective manage- 127  
ment practices to ensure better yields of ‘Rossa di 128  
Tropea’. Due to the different features of these two 129  
viruses, two parallel and independent approaches were 130  
used, starting from surveys to assess the incidence of 131  
OYDV and IYSV in different production phases by 132  
visual inspection and laboratory assays. Moreover, for 133  
OYDV, some disease aspects such as virus presence in 134  
various plant parts, and virus transmission through seed 135  
in cv. Rossa di Tropea, including the evaluation of seed 136  
production losses, were assessed. For IYSV, due to a 137  
long debated hypothesis about its presence in bulbs, 138  
experiments were carried out to determine if infected 139  
bulbs constitute an internal infection source in the field. 140

## 141 Materials and methods

### 142 Monitoring OYDV and IYSV incidence

143 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 145  
out in a farm where biennial cycle of onion production 146  
was followed (year 1 from seed to bulb; year 2 from 147  
bulb to seed). In particular, year 1 (bulb production) 148  
starts in September with sowing in seedbeds in an open 149  
field, followed by transplantation of seedlings in 150  
November–December and bulb harvesting in June. Af- 151  
ter three months of storage in a warehouse, bulbs are 152  
transplanted in October of year 2 (seed production) and 153  
the seeds harvested in early July (Supplementary Fig. 1). 154

155	During our survey seedbeds were in open field and no	100% of suspected IYSV symptomatic plants since	201
156	other onion crop was cultivated in close proximity,	lesions caused by this virus could be confused with	202
157	except in 2012 when a green onion crop was grown.	those produced by other biotic or abiotic agents.	203
158	Bulb production was accomplished in the same area of		
159	seedbeds near the sea level, whereas seed production	<i>Green onion</i> Since a green onion cultivation was adja-	204
160	was carried out in an area 200 m above sea level, higher	cent to the 2012 seedbeds, this crop was surveyed for	205
161	than the other onion fields. Fertilization, and fungicide	OYDV and IYSV incidence for symptoms followed by	206
162	and pesticide treatments were as prescribed for the	laboratory testing of 90 plants.	207
163	'Rossa di Tropea' onion PGI production regulation. A		
164	complete biennial cycle (2012–2014) and two incom-	Distribution of OYDV in plant and seed-transmission	208
165	plete cycles (2012–2013 and 2013–2014) were moni-		
166	tored and compared to the growing stages. Methods of	The distribution of the virus was recorded in ten infected	209
167	sampling were chosen according to plant growth stage	plants collected from 2013 bulb crop by testing for virus	210
168	and detection assays as described below.	presence: roots, all bulb portions (outer tunic and inner	211
		scales, basal plate and vegetative apex) and all leaves.	212
169	<i>Seedlings</i> Before transplanting, more than 300	Furthermore, ten infected plants from 2014 seed crop	213
170	seedlings/year (15–20 cm tall) were randomly collected	were analyzed in inflorescence stems and in floral en-	214
171	from seedbeds and pooled (10 seedlings per pool), then	velopes. OYDV presence was also tested in seeds by	215
172	examined for virus presence by molecular assays. In	analyzing a stock of 200 seeds per year (20 samples of	216
173	cases where the pooled sample tested positive, seedlings	ten seeds each) from the 2012 and 2013 crop and 40	217
174	from that pool were individually tested to determine the	seeds (individually tested) from the 2014 crop. Seeds	218
175	ratio of infection over the total number of sampled	from 2012 and 2013 were provided by the farm and	219
176	seedlings.	were collected without distinguishing if they originated	220
		from infected or healthy plants, whereas the seeds in	221
177	<i>Dormant bulbs</i> Bulbs were randomly collected from the	2014 were all collected from infected plants. Seed trans-	222
178	storage warehouse in September 2012 and 2013. Each	mission of OYDV in 'Rossa di Tropea' onion was	223
179	bulbs sample was split into two groups: part of the bulbs	examined by sowing 300 seeds from each stock of	224
180	was used to determine the presence of OYDV and IYSV	2012 and 2013, and 400 seeds of 2014 production.	225
181	in apex tissues, and the other part was placed in pots,	The experiment was carried out in an insect-proof green-	226
182	grown in an insect-proof greenhouse, and the emerged	house and seedlings were tested when were 15 cm tall.	227
183	plants were tested for the presence of both viruses (the	Samples were pooled in groups of five individuals.	228
184	number of tested bulbs for each year and the virus		
185	infection are reported in Tables 2 and 4). Since IYSV	OYDV influence on seed production	229
186	was absent and OYDV-infection was very low in plants		
187	from the 2014 bulb crop, 90 bulbs collected in Septem-	The effect of OYDV on seed production was assessed by	230
188	ber 2014 were planted in a greenhouse and the resulting	monitoring plants ( $N=300$ ), from early stage to seed	231
189	plants were tested only for OYDV.	maturity. The assessment was done during the 2014 seed	232
		crop. Plants from this seed crop were also surveyed in	233
190	<i>Plants in the field</i> Three plots/year of 500 plants/plot in	July 2014. Four categories of damage were used for	234
191	bulb crop and 100 plants/plot in seed cultivation, re-	rating the disease severity of plants: (1) those that died	235
192	spectively were randomly chosen in the field to deter-	soon after sprouting, (2) infected and unproductive, (3)	236
193	mine the plants' phytosanitary status. A total of 1500	early-infected but productive (OYDV positive in May),	237
194	and 300 plants were monitored for virus symptoms	and (4) late-infected (OYDV positive in July). The	238
195	twice/year, in April and in May–June, before the har-	number and the weight of seeds/inflorescence, and the	239
196	vesting. During the visual monitoring of symptoms, leaf	weight of 100 seeds/inflorescence were determined in	240
197	samples from symptomatic plants were collected to test	seeds collected from 13 plants of each productive group	241
198	virus presence by laboratory assays. In particular, leaves	(3 and 4). Univariate analysis of Variance	242
199	were collected from 10 to 30% of OYDV symptomatic	(UNIANOVA), following the general linear model	243
200	plants to confirm the visual detection, whereas from	(GLM) procedure, was performed with the two	244

245	productive groups (3 and 4) as fixed factors and the	100 µL of the macerate were used for total RNA extrac-	289
246	number of seed/inflorescence, the total weight of	tion. RNA extracted was eluted in 100 µL of nuclease-	290
247	seed/inflorescence and the weight of 100 seeds as de-	free water supplied with the kit.	291
248	pendent variables. No statistical transformation was		
249	needed because the ANOVA assumptions (homogeneity	<i>Single step RT-PCR</i> Reverse transcriptase polymerase	292
250	and normality of variance across the groups) were not	chain reaction (RT-PCR) was used for: i) OYDV detec-	293
251	violated (Levene's and Shapiro Wilk test $p > 0.05$ ). Data	tion in dormant bulbs, seeds and seedlings with primers	294
252	analysis was performed by means of R version 2.3.0 (R	OYDV-NIb/CP F1 and OYDV-NIb/CP R1 that amplify	295
253	Development Core Team 2010).	a 984 bp fragment, which includes part of nuclear in-	296
		clusion B (NIb) and part of coat protein (CP) of the virus	297
254	IYSV presence in bulb and bulb transmission	(Manglli et al. 2014); and ii) IYSV detection in fresh	298
		plant material with oligonucleotides IYSV-Nc5 and	299
255	Part of this aim of research was studied by examination	IYSV/Nc3 targeting a 614 bp fragment in the nucleo-	300
256	dormant bulbs randomly collected as above described	protein (N) gene (Tomassoli et al. 2009). All single-step	301
257	(see Monitoring virus incidence: <i>Dormant bulb</i> ). In	RT-PCRs were performed in a total volume of 25 µL	302
258	addition, the migration of IYSV from infected leaves	that consisted of 2 µL of Total RNA extract, 1X PCR	303
259	to bulbs was studied by testing the presence of IYSV in	buffer (Promega, Madison, WI, USA), 2.5 mM of each	304
260	the apex of 12 fresh bulbs collected from infected but	dNTP, 4 µM of sense and antisense primers, 1.2 U	305
261	still green plants (just before harvesting time). Subse-	AMV-RT (Promega), 20 U RNase-OUT (Invitrogen,	306
262	quently, to address whether the virus is transmitted from	Carlsbad, CA, USA), 0.75 U GoTaq Polymerase	307
263	the bulb to the emerging plant, 22 IYSV-infected plants	(Promega), in a final volume with RNase-free water.	308
264	were collected from the bulb crop. Plants were left until	Amplification cycles were: 46 °C for 30 min for the	309
265	the leaves had dried, and the bulbs were stored for three-	cDNA synthesis, followed by 5 min at 95 °C for dena-	310
266	months, then were planted in an insect-proof green-	uration and 35 cycles of amplification (for OYDV:	311
267	house. The plants from these bulbs were observed for	1 min at 94 °C, 1 min at 57 °C and 1 min at 72 °C; for	312
268	symptoms and tested at different growing stages up to	IYSV:45 s at 94 °C, 45 s at 58 °C and 45 s at 72 °C), and	313
269	inflorescence formation.	a final extension at 72 °C for 10 min. RT-PCR products	314
		were visualized in 1.2% agarose gel by ethidium bro-	315
270	OYDV and IYSV laboratory assays	midide (EtBr) staining.	316
271	<i>Serological assay</i> DAS-ELISA (Clark and Adams	<i>Real-time RT-PCR</i> The method was applied to deter-	317
272	1977) was used for OYDV as a detection test after visual	mine the presence of IYSV in dormant bulbs, in plantlets	318
273	diagnosis in the field. Commercial kits (Loewe Ghm,	generated by the bulbs, and in fresh bulbs obtained from	319
274	Sauerlach, Germany and Bioreba Reinach, Switzerland)	IYSV infected plants. Oligonucleotides (IYSV	320
275	were used following the manufacturer's instructions.	432F/503R) and probe (IYSV 455) were previously	321
276	ELISA reactions were measured by a spectrophotometer	described by Tiberini et al. (2012) (Table 1). The	322
277	Multiscan FC (Thermo Scientific) at 405 nm and the	TaqMan probe was labeled with the 6-FAM fluorophore	323
278	samples were considered OYDV-positive when absor-	and quencher BHQ (Applied Biosystems, Life Technol-	324
279	bance readings values were at least twice greater than	ogies; MGB from Sigma Company).	325
280	the average absorbance readings of the healthy control.		
		The reaction mixture (25 µL) contained 12.5 µL	326
281	<i>Total RNA extraction</i> Total RNA was extracted using	Taqman 2 X universal PCR Master Mix, 0.625 µL	327
282	"Real Total RNA from Tissue and Cell" kit (Durviz,	Multiscribe RNase Inhibitor Mix (Applied Biosystems,	328
283	Valencia, Spain) following the manufacturer's instruc-	Life Technologies), 0.75 µL (10 µM) of each primer,	329
284	tions. Approximately 0.5 g of plant tissue were used.	0.5 µL (5 µM) TaqMan probe, 7.875 µL RNase-free	330
285	The vegetative apex of the bulbs, part of seedlings after	water, and 2 µL of total RNA extract. The amplification	331
286	emergency, and part of the plant leaves were macerated	reaction was carried out in a 7500 Fast Real Time	332
287	1:5 (w:v) in phosphate buffer. For seed assays the mac-	Thermocycler (Applied Biosystems) using 48 °C for	333
288	eration ratio was 1 seed in 100 µL of phosphate buffer.	30 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



336 elongation at 60 °C for 1 min. Positive, negative and  
337 internal controls (5.8 s rRNA gene) (Robene et al.  
338 2015), were included. Results were visualized and ana-  
339 lyzed by SDS software (Applied Biosystems, Life Tech-  
340 nologies). The test was duplicated for each of the  
341 samples.

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

350 The reaction mixture (20 µL) for nested PCR  
351 contained 1X PCR buffer (Promega, Madison, WI,  
352 USA), 2.5 mM of each dNTP, 4 µM of sense and  
353 antisense primers, 0.75 U GoTaq Polymerase  
354 (Promega), 2 µL (undiluted) of the RT-PCR product  
355 and RNase-free water. The amplification cycle was per-  
356 formed with an initial denaturation at 95 °C for 3 min,  
357 followed by 35 cycles at 94 °C for 30 s, 58 °C for 30 s,  
358 72 °C for 1 min, and a final extension at 72 °C for 5 min.  
359 Nested PCR amplicons were visualized in 1.2% agarose  
360 gel after EtBr staining. Data were compared with the  
361 results obtained in real-time RT-PCR and only the sam-  
362 ples that amplified in both methods were considered  
363 positive.

## 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)

368 which subsequently increased to 6.40% (96/1500) and  
369 reached 36.20% (543/1500) by the time the crop was  
370 ready for harvesting. Symptoms included dwarfing of  
371 plants, leaf flattening, crinkling, and yellowing (Fig. 1a).  
372 In year 2, stored bulbs had 17.86% (25/140) OYDV  
373 infection which subsequently had increased to 98.67%  
374 in the seed crop by May 2014 (Table 2). At this devel-  
375 opment stage, severe flower stem distortion and curling  
376 were observed (Fig. 1b). The green onion crop near to  
377 the seedbeds had 90% of OYDV infected plants. In year  
378 2 of cycle 2011–2013, the initial OYDV infection of the  
379 onion seed crop was 15.87% (10/63) infected bulbs,  
380 which increased to 91.34% (274/300) of OYDV inci-  
381 dence by June 2013. In year 1 of the cycle 2013–2015,  
382 0.83% (3/360) of bulb crop seedlings was infected, and  
383 in April 2014 only two symptomatic plants out of 1500  
384 (0.13%) were found and were eradicated. In May 2014,  
385 no virus-associated symptoms were detected and testing  
386 showed only one infected sample from the surveyed  
387 plot. Moreover, bulbs collected randomly from the  
388 warehouse in September 2014 did not generate OYDV  
389 infected plants (0/90).

### Distribution of OYDV in plant and seed-transmission 390

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

400 RT-PCR testing of seeds from 2012 and 2013 (200  
401 seeds/year, 20 samples of 10 seeds each) were all posi-  
402 tive 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'-GCTTCCTCTGGTGAGTGAT-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



403 from infected plants and tested, 22 out of 40 were  
 404 positive for OYDV. On the contrary, virus infection  
 405 was not found in 599 seedlings grown from seeds in  
 406 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  
 413 as the total weight of seeds produced per inflorescence  
 414 obtained from 13 early-and 13 late -infected plants

(categories 3 and 4 respectively) were statistically dif- 415  
 ferent ( $F = 84.1$  and  $91.5$  respectively,  $df = 1, p < 0.001$ ). 416  
 However, averages of 100 seeds' weight of groups 3 and 417  
 4 and did not differ significantly ( $F = 0.243$ ,  $df = 1, p =$  418  
 $0.626$ ) (Table 3). 419

IYSV incidence 420

No IYSV infection was found in 340 and 360 seedlings 421  
 collected in years 2012 and 2013 respectively. The virus 422  
 was detected at low percentage (1.49% and 3.57%) in 423  
 bulbs randomly collected at the warehouse, however, 424  
 IYSV infection in plants growing from bulbs in the 425  
 greenhouse was 0%. During the three monitored years 426

Q4:2.1 **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

symptomatic/total plants present in selected areas), confirmed by DAS-ELISA analysis

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

Nm: not monitored; <sup>a</sup> apex of dormant bulbs and <sup>b</sup> 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

427 in both growing stages, IYSV infection did not exceeded  
 428 3.33% (Table 4) at the end of cultivation cycle. Foliar  
 429 symptoms associated with IYSV consisted of small or  
 430 large chlorotic and necrotic spots (Fig. 2), forming char-  
 431 acteristic diamond-shaped lesions, single or concentric,  
 432 which were evident on flower stem (Fig. 3) of seed crop.  
 433 IYSV infections were detected visually and confirmed  
 434 by single-step RT-PCR analysis earlier in seed than in  
 435 bulb crop. In fact, 1% and 0.33% IYSV infection were  
 436 detected in April 2013 and 2014 in seed crops, respec-  
 437 tively, while in bulb crop, IYSV infection was found  
 438 later in June. Moreover, the final infection rate was  
 439 higher in seed crop (3.33% in 2013 and 2.67% in  
 440 2014) than in bulb crop (0.87% in 2013 and 0% in  
 441 2014). No IYSV infected plants were detected in the  
 442 green onion lot near to the seedbeds during cycle 2012–  
 443 2014.

#### 444 IYSV presence in bulb and bulb transmission

445 In the context of the diagnostic assays carried out  
 446 to examine the virus incidence in stored randomly  
 447 collected bulbs, real-time RT-PCR and nested PCR  
 448 were carried out. Real-time RT-PCR results  
 449 showed the presence of IYSV in the vegetative  
 450 apex of 15 out of 123 bulbs and in two out of  
 451 109 plants growing from other mother bulbs  
 452 transplanted into a greenhouse. Samples yield am-  
 453 plification signals after 37th–38th reaction cycles,  
 454 in both of the duplicated samples. Contrarily, the  
 455 healthy and the majority of unknown field samples  
 456 did not showed any target amplification. When  
 457 nested PCR was used to test those that were  
 458 positive in real-time RT-PCR, only 3/15 and 0/2  
 459 gave amplicons of expected size (Table 5). When  
 460 apexes of 12 fresh bulbs of infected, but still  
 461 green plants were tested, RT-PCR did not show  
 462 amplification of IYSV (0/12), whereas the virus  
 463 was detected both by real-time RT-PCR and by  
 464 nested PCR (12/12). To determine if the virus is  
 465 transmitted from bulb to the new plant, the  
 466 resulting plants from other 22 bulbs from IYSV-  
 467 infected plants, stored and then transplanted in the  
 468 greenhouse tested all negative for IYSV in differ-  
 469 ent stages of the growth. Several plants showed  
 470 chlorotic/necrotic spots that were identified to be  
 471 caused by *Alternaria* spp. and/or *Peronospora*  
 472 *destructor* (, Rome, Italy).

## 473 Discussion

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

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

501 It is important to highlight, that during the complete  
 502 biennial cycle (2012–2014) a high infection of OYDV  
 503 was detected, and a green onion crop highly infected  
 504 with OYDV (about 90%) was growing near the seed-  
 505 beds. It appears that similar situation of green onion crop  
 506 in close proximity had existed in the previous cycle  
 507 (2011–2013). It should be noted that years 2 had  
 508 91.34% OYDV infection in June. When the location of  
 509 seedbeds was farther from the green onion, year 1 of last  
 510 cycle (2013–2015) had 0.07% of OYDV incidence in  
 511 the bulb crop and the absence of OYDV infection in  
 512 stored bulbs collected in September 2014 (Table 2). Our  
 513 results support that the physical isolation of seedbeds  
 514 from infected crops are the most important management  
 515 measures, and are in agreement with a previous study  
 516 reporting a progressive declining of OYDV incidence  
 517 with an increasing distance from the virus inoculum  
 518 source (Ahmed and Elhassan 2013).

519 The importance of keeping the different production  
 520 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

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

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)

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

533 The effect of OYDV infection on seed production  
 534 was determined during the cycle 2013–2014. Infected  
 535 bulbs caused quick decline of plants and resulted in 31%  
 536 loss of productive plants. The negative effect of OYDV  
 537 on seed production included reduction of weight and  
 538 number seeds per inflorescence (45.31% and 44.23%,  
 539 respectively), which was in agreement with previous

540 reports (Elnagar et al. 2011; Kumar et al. 2012). How-  
 541 ever, there was no significant difference in the specific  
 542 weight per seed between the two analyzed groups of  
 543 plants, suggesting that there was no qualitative damage  
 544 due to different times of infection. These results, even  
 545 though not compared with healthy plants, were in close  
 546 agreement with Rudolph (1990) who described signifi-  
 547 cant decline of OYDV infection in seed yield, but not in  
 548 seed quality.

549 It is important to underline that 599 seedlings gener-  
 550 ated from seeds collected in our survey turned out to be  
 551 virus free, indicating there was no viral seed transmis-  
 552 sion in ‘Rossa di Tropea’. These results are in accor-  
 553 dance with data previously reported (Bos 1976), and  
 554 differ from data by other authors (Abd El-Wahab et al.  
 555 2009) who reported OYDV transmission by seed in two  
 556 Egyptian onion cvs based on OYDV detection in seed.  
 557 Our results showed no transmission in grow out tests,  
 558 although 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 select-  
 ed areas), confirmed by single step RT-PCR

Biennial cycles	Year 1 - Bulb production			Year 2 - Seed productions		
	Seedlings	Plants	Plants	Dormant Bulbs	Plants	Plants
2011–2013	Nm			20-Sept-‘12	16-Apr-‘13	3-Jun-‘13
Infected/total observed				1 <sup>a</sup> /67	0 <sup>b</sup> /69	3/300
Infection rate (%)				1.49	0	1
2012–2014	8-Nov-‘12	16-Apr-13	03-Jun-‘13	11-Sep-‘13	13-Apr-‘14	13-May-‘14
Infected/total observed	0/340	0/1500	13/1500	2 <sup>a</sup> /56	0 <sup>b</sup> /40	1/300
Infection rate (%)	0	0	0.87	3.57	0	0.33
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; <sup>a</sup> apex of dormant bulbs and <sup>b</sup> 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

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

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

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

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

588 The reappearance of IYSV, although at low levels, in each production cycle remains as an epidemiological  
 589 question for further study. While IYSV is not transmitted by seed (Kritzman et al. 2001; Bulajić et al. 2009),  
 590 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 yellow spot tospovirus detection in randomly collected dormant bulbs by real-time RT-PCR and nested PCR

Year of detection	2012		2013	
	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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