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Research Article

Garlic (*Allium* spp.) viruses: detection, distribution and remediation attempts in a European garlic collection

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Abstract

Garlic is an important vegetable crop in numerous countries used as food and natural based medicine. Similar to the majority of vegetatively propagated plants, garlic may be affected by several viruses that can cause severe crop losses. The present study aimed to screen 105 garlic accessions (mother plants) from 5 European countries (Germany, Czech Republic, Poland, Italy, and France) for possible presence of *Onion yellow dwarf virus* (OYDV), *Leek yellow stripe virus* (LYSV), *Garlic common latent virus* (GCLV) and *Shallot latent virus* (SLV). The occurrence of three *Allexiviruses* (GarV-A, GarV-B and GarV-C) in mixed assays was also investigated. Meristem-tip culture assays were performed in order to attempt eradication of the studied viruses. Garlic viruses identification was made by ELISA and RT-PCR. ELISA outcomes showed that all 105 garlic accessions were infected by different virus combinations. The OYDV and LYSV were identified, by ELISA, in all countries at 96% and 88,6% respectively and by RT-PCR at 99% and 96%. Furthermore, GCLV and SLV were detected by ELISA in about 88% and by RT-PCR at 89% and 90%, respectively with the exception of the studied *Allexiviruses* which were not amplified by RT-PCR with ALLEX1/ALLEX2 primers. Smaller meristem size (0,3-1,5 mm) led to better virus elimination efficiency (29%) compared to 8% obtained for the larger size (2-2,5 mm). The outcomes were opposite (16% vs. 90%) for plants regeneration. Virus elimination efficiency was linked to the virus type, e.g., OYDV and LYSV were eradicated at 90% while GCLV and *Allexiviruses* were difficult to eliminate (57,4% and 55,6% of eradication). Given the economic relevance of garlic crops worldwide and the frequently reported incidence of viral infections, it is important to make virus-free germplasm available. Therefore, investigating the garlic germplasm sanitary status and constantly improving it is of crucial importance aiming to increase the overall garlic production.

Keywords: *Allium*; ELISA; meristem; RT-PCR; viruses

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Introduction

Garlic is an herbaceous, perennial bulbous plant originating in West-Central Asia. It has been known for centuries and is currently being cultivated worldwide for its aromatic, medicinal and therapeutic properties making it economically important. The colonization and adaptation of this plant to various habitats promoted an extremely high morphological diversity related to bulb dimension, foliage vigour and the size of the inflorescence. Within the species, this diversity is demonstrated by the presence of five taxonomic groups: *Longicuspis*, *Pekinense*, *Ophioscorodon*, *Sativum* and Subtropical (Maaβ and Klass, 1995). Fritsch and Friesen stated that garlic is the second most important species in *Allium* genus (Fritsch and Friesen, 2002).

World garlic production, in 2020, was around 28 million t cultivated on 1.63 million ha (FAOSTAT, 2020). Among the most important producing countries are China (20,757,034 t), India (2,917,000 t), Bangladesh (485,447 t) and South Korea (363,432 t). In 2020, FAOSTAT data reported that the European Union (EU) countries produced 867,275 t of garlic on 102,824 ha. Among these countries, Spain was predominant with 269,090 t, followed by Italy with 29,270 t, France with 22,430 t, Romania with 27,590 t and Poland with 13,500 t (FAOSTAT, 2020). The first exporting country is China with 2,254,201 t of garlic with a profit of about 2 million dollars. The high quality and the low price of Chinese garlic often makes the cultivation of this crop in many European countries uncompetitive. In fact, the EU, with 341,715 t of garlic purchased annually, is one of the top importers (FAOSTAT, 2020). Other factors have also contributed to the progressive decrease of garlic cultivation in Europe and, among them, the most important one is the phytosanitary problem of this crop. In particular, viral diseases often occur due to its obligated vegetative propagation. In addition, garlic mother plants used for propagation could be infected by one or multiple viruses and during the repeated vegetative cycles often there is an accumulation of viruses which has negative effects on the production and the quality of the bulbs (Verhoyen and Horvat, 1973; Walkey, 1990; Kereša *et al*., 2021). The percentage of virus infection in garlic generally oscillates from 40% to 74% sometimes reaching 100% (Van Dijk, 1993; Guenaoui *et al*., 2013; Gutiérrez *et al*., 2021). Garlic can be infected by many viruses mainly belonging to the *Potyvirus*, *Carlavirus* and *Allexivirus* genera which can severely affect the plants in fields with important production losses (Barg*et al*., 1997). The most common viruses found in garlic and causing major damage are members of the *Potyvirus* family like *Onion Yellow Dwarf Virus* (OYDV) and *Leek Yellow Stripe Virus* (LYSV), the two *Carlaviruses*, *Shallot Latent Virus* (SLV) and *Garlic Common Latent Virus* (GCLV) along with *Allexiviruses*, *Slallot Virus X* (ShVX) and GarV-A, Garv-B; GarV-C, GarV-D and GarV-X. The OYDV and LYSV have been reported in some species of the *Alliaceae* family such as garlic, onion and leek (Barg *et al*., 1997; Lot *et al*., 1998; Tsuneyoshi *et al*., 1998; Harti *et al*., 2020; Cremer *et al*., 2021). Symptoms include chlorotic stripes and leaf malformation or deterioration of the flowers (Conci *et al*., 2003). *Carlaviruses* GCLV and SLV have proved to infect many plants of the *Allium* Genus and causing chlorotic stripes on leaves leading also to relevant economic damage (Conci *et al*., 2003). Moreover, the OYDV, LYSV, GCLV and SLV have been described and reported to cause infections in cultivated garlic plants or in garlic germplasm collection in Czech Republic, Italy, France, Germany, and Poland (Klukáčková *et al*., 2007; Smékalová *et al*. 2010; Dovas and Vovlas, 2003; Botti *et al*., 2003; Messiaen *et al*., 1994; Delecolle and Lot, 1981; Barg, 1996; Graichen, 1976; Sala-Rejczack *et al*., 2012; Chodorska *et al*., 2014). The damage caused by these viruses could lead to 50% losses of the entire production (Canavelli *et al*., 1998) and to a reduction of bulb weight ranging from 24% to 70% (Messiaen *et al*.,1994; Lunello *et al*., 1999; Bagi *et al*., 2012). In addition, complex viral infections cause further major reductions of garlic production (Klukáčková *et al*., 2007, Bagi *et al*., 2012; Lunello *et al*., 2007). The *Allexiviruses* are generally asymptomatic, causing less damage to the host plants and often found in mixed infections (Dovas *et al*.; 2001; Chen *et al*., 2001; Mohamed *et al*., 2013; Paranno *et al*., 2012). Data about the distribution of *Allexiviruses* in European countries is scarce (Sala-Rejczak *et al*., 2012; Bereda *et al*., 2017).

In Europe, numerous garlic accessions from Czech Republic, Germany, Poland, France and Italy, with valuable morpho-productive characteristics, are preserved in the European *Allium* Database (EADB). In order to conserve virus-free garlic plants and help to commercialize virus-free material, the EU financed the European Alliums Vegetatively Propagated - "EURALLIVEG" project. The present work intended to analyse the garlic accessions belonging to the European collections (EADB) with the aim to: i) verify the presence and distribution of the most common garlic viruses in mother plants originated from 5 European countries by Enzyme-linked Immuno-Sorbent Assay (ELISA) and RT-PCR; ii) attempt to obtain virus-free garlic plants through meristem-tip culture and evaluate the efficiency of virus remediation also in order to have information regarding difficult virus eradication.

Materials and Methods

Biologic material

The biological material investigated in this study was selected from EADB based on its elevated cultural characteristics and consisted in 105 valuable accessions (mother plants) originated from Germany (31), Poland (31), Czech Republic (19), France (5) and Italy (19). Each garlic accession under study was composed of five plants and analysed in the Plant Pathology Laboratory (PPL) at the School of Agricultural, Food and Environmental Sciences (SAFE) of the University of Basilicata, Italy.

Serological analyses

All the samples (5 samples/accession) were analysed by Double Antibody Sandwich Enzyme-Linked-Immuno-Sorbent Assay (DAS-ELISA) (Clark and Adams, 1977) to determine the presence of OYDV, LYSV, GCLV, SLV, GarV-A, Garv-B and Garv-C. SLV BIOREBA (BIOREBA AG, Reinach, Switzerland) kits were used for OYDV, LYSV, GCLV. The DSMZ kit (DSMZ, Braunschweig, Germany) was used for *Allexiviruses* (GarV-A, Garv-B and Garv-C) which detects the above virus complex without distinction.

From each plant 1g of leaf tissue was homogenized in 10 mL of extraction buffer furnished with the ELISA kit and 200 µL of the extracted sample was subsequently processed in Nunc™ MicroWell 96- Well Microplates (ThermoFisher Scientific Inc., Waltham, MA, U.S.A.) following the manufacturer's protocol. The plates were read using an ELISA Reader model A3 (DAS, Rome, Italy) at 405 nm wavelength.

Molecular analyses

Furthermore, for all viruses molecular investigations by RT-PCR following a protocol of Meenakshi et al. (2006) were performed. A total of 100 mg of leaf tissue was used to extract the total RNA (tRNA) with the RNA Plant Mini Kit Qiagen (Hilden, NRW, Germany) following the manufacturer's instructions. A total of 500 ng of tRNA was used in the RT-PCR reaction utilizing the kit SuperScript IV One-Step RT-PCR System with Platinum® SuperFi DNA Polymerase (Invitrogen, ThermoFisher Scientific, Waltham, MA, U.S.A.) for the cDNA production following manufacturer's instructions and subsequently used for PCR amplification which was carried out as described by Altieri *et al*. (2010).

Four separate pairs of primers OYDV1/OYDV2, LYSV1/LYSV2, GCL-N30/GCL-C40, SL-N30/SL-C10 taken from the literature (Dovas *et al*., 2001; Nam *et al*., 2015) were used. For the *Allexiviruses*, GarV-A, GarV-B, GarV-C and GarV-D, primers ALLEX1/ALLEX 2 from the literature were used. Details of the RT-PCR primers are presented in Table 1.

Table 1. Primers used in RT-PCR for the detection of garlic viruses

Virus	Primer	Sequence 5'→3'	Target Gene*	Amplicon size (bp)	Reference
OYDV	**OYDV 1**	GAAGCACAYATGCAAATGAAGG	CP	283	[Dovas *et al.*, 2001]
	OYDV2	GCCACAACTAGTGGTACACCA			
LYSV	**LYSV 1**	CACATCAAGAACACCAGTTAGAGC	CP	304	[Dovas *et al.*, 2001]
	LYSV 2	GTAGAAACTGCCTTGAACGAGTG			
GCLV	**GCL-N30**	GCACCAGTGGTTTGGAATGA	CP	481	[Nam *et al.* 2015]
	GCL-C40	AGCACTCCTAGAACAACCATTA			
SLV	**SL-N30**	TATGGTAACGAAGAAGAAGAACTC	CP	203	[Nam *et al.*, 2015]
	SL-C10	CGTTCACGCTAGACAATTCAGACAT			
GarV-A, GarV-B, GarV-C, GarV-D	**ALLEX 1**	CYGCTAAGCTATATGCTGAARGG	ORF VI	183-192	[Dovas *et al.*, 2001]
	ALLEX 2	TGTTRCAARGTAAGTTTAGYAATATCA ACA			

*Notes:CP= coat protein; ORF VI= open reading frame VI of the *Allexiviruses*. Degenerated bases: Y= T, C; R=A,G

Five µl of each PCR product was verified by horizontal electrophoresis on a 1.2% agarose gel run in 1X TAE buffer and visualized by UV transilluminator (Euro Clone S. p. A., Pero, Milan, Italy) after coloration with SYBR Safe DNA Gel stain (ThermoFisher Scientific, Waltham, MA, U.S.A). A 1kb DNA ladder (Invitrogen, ThermoFisher Scientific, Waltham, MA, U.S.A.) was also used as a marker.

Meristem culture

Meristem preparation

From each garlic accession, 50 apical meristems (from a total of 2625 obtained), previously tested for the presence of the investigated viruses by ELISA and RT-PCR, were taken under aseptic conditions using a laminar hood. In order to check the involvement of the meristem size on the remediation efficiency, two different types of meristems were considered. Half of the meristems were taken with only one foliar primordia (0,3-1,5 mm) named small meristem (SM) and the other half was taken with more primordia (2-2,5 mm) named large meristem (LM). The external covering parts of the garlic bulbs and cloves were taken off and they were sterilized with ethanol 70% and sodium hypochlorite 50% and finally rinsed twice with sterile water and dried on sterile paper. The biologic material was kept at 8 °C and utilized within 2 days from the preparation.

In vitro culture of garlic meristems

Excision of the meristems took place in aseptic conditions under a laminar hood with a flame and also with the help of a stereomicroscope with optical fibre with cool light model Zeiss (40x) (Karl Zeiss, Dresden, Germany). All apical meristems were placed in sterile Nunc™ Cell Culture Treated Multidishes (ThermoFisher Scientific, Waltham, MA, U.S.A.) on Murashige Skoog (MS) media (Murashige and Skoog, 1962) with the hormones kinetin (KIN) (Sigma Aldrich, Darmstadt, Germany) and 3-Indoleacetic acid (IAA) (Sigma Aldrich, Darmstadt, Germany). Subsequently, they were placed in a growth chamber under controlled light

conditions (16 h- 4000 Lux) at 18±1 °C for a period ranging from 6 to 12 weeks depending on the accession *in vitro* culture requirements. The obtained plantlets (of about 1-2 cm long) were transferred on growth MS media supplemented with 1-Naftaleneacetic acid (NAA) (Sigma Aldrich, Darmstadt, Germany) and N6-(2-isopentenyl) Adenine (2iP) (Sigma Aldrich, Darmstadt, Germany) until they reached 8-9 cm in length which was the useful dimension to be used in the subsequent analysis.

Evaluation of viruses remediation

In order to evaluate virus remediation, the 2780 plantlets obtained were sectioned horizontally under sterile conditions and the basal part was sown in MS tubes while the apical one was homogenized and further processed by ELISA and RT-PCR. The multiplication *in vitro* was done only for the virus-free garlic accessions. In order to verify the remediation percentage of each single virus, 50 plantlets for LM and 50 for SM, infected by all viruses investigated, were analysed by RT-PCR.

Results

Serological analyses

Results from this study demonstrated that in the 105 garlic accessions analysed (5 plants/accession) the OYDV, LYSV, SLV and GCLV had been detected in all the garlic accessions investigated with a variable incidence ranging from 78% to 100%. Furthermore, garlic accessions originated from France and Czech Republic were also found to be 100% infected by the *Allexiviruses* studied (GarV-A, GarV-B and GarV-C). These last viruses were detected with an incidence ranging from 21% (Germany) to 81% (Italy) (Figure 1). All garlic accessions analysed resulted infected at least by two viruses. No virus-free garlic mother plants were found.

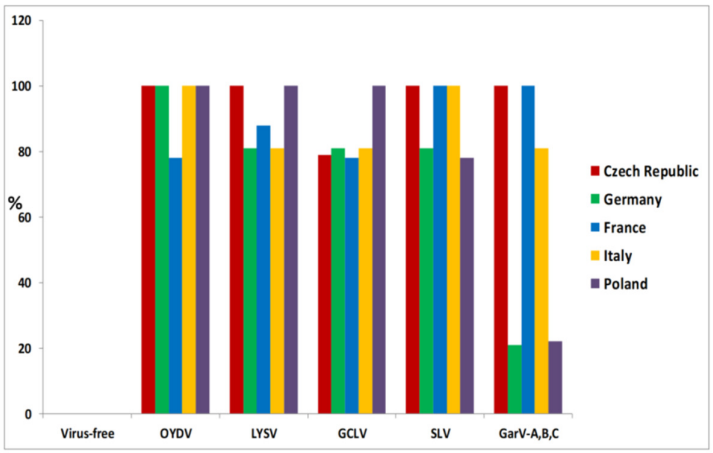

Figure 1. Percentage of infection for each virus in the 105 European garlic accessions divided by origin

In addition, garlic accessions analysed in this study showed mixed viral infections. The percentages of mixed virus infection ranged between 21% to 79% (Czech Republic), 19% to 23% (Germany), 18% to 60% (France), 19 to 59% (Italy) and 22% to 78% (Poland). Distribution of the investigated viruses in mixed infections for each country was various and details about the number and type of viruses found are presented in Table 2.

Table 2. Garlic virus distribution in mixed infections for each of the 5 European countries investigated in this study

Viral Infection (%)**						
No. of viruses*	**Viruses in mixed infections**	**Czech Republic**	**Germany**	**France**	**Italy**	**Poland**
2/5	OYDV + LYSV	0	23	0	0	0
3/5	OYDV + GCLV + SLV	0	19	0	19	0
3/5	OYDV + LYSV + GCLV	0	0	0	0	0
3/5	LYSV + SLV + GarV-A, B, C	0	0	22	0	0
4/5	OYDV + GCLV + SLV + GarV-A, B, C	0	0	18	0	0
4/5	OYDV + LYSV + SLV + GarV-A, B, C	21	0	0	22	0
4/5	OYDV + LYSV + GCLV + GarV-A, B, C	0	0	0	0	22
4/5	OYDV + LYSV + SLV + GCLV	0	37	0	0	78
5/5	OYDV + LYSV + GCLV + SLV + GarV-A, B, C	79	21	60	59	0

Notes: **Allexiviruses* (GarV-A, B, C) were counted together. **Percentages of viral infection found in each country were approximated to entire numbers.

Molecular analyses

The outcomes of the RT-PCR performed on the 105 garlic accessions investigated in this study, as expected, did not confirm entirely to the ELISA assay results. In particular, from the 105 garlic accessions investigated by ELISA, about 4% in the case of OYDV and 11.4% in the case of LYSV resulted negative, while when tested by RT-PCR only 1% for OYDV and 4% for LYSV were found virus-free. Amplicons of expected sizes (Table 1) were obtained with almost all primer pairs used except for ALLEX1/ALLEX2 primers. Negative controls did not show any amplicons. The obtained amplicons were directly sequenced by BMR Genomics (Padua, Italy). Nucleotide sequences, for all viruses, were compared to those already present in GenBank showing a high similarity (>94%) with nucleotide sequences for the same viruses.

Meristem cultures

After 6-12 weeks from the apical meristems placement on MS media, a very high percentage of rooting (90%) was observed in the case of the meristems with sizes ranging from 2 to 2.5 mm while for the smaller ones (0.3-1.5 mm) only a 16% of rooting was obtained. From a total of 2625 explants done in this study for each of the two groups (SM and LM), 2360 plantlets were obtained for the LM and 420 for the SM (Figure 2).

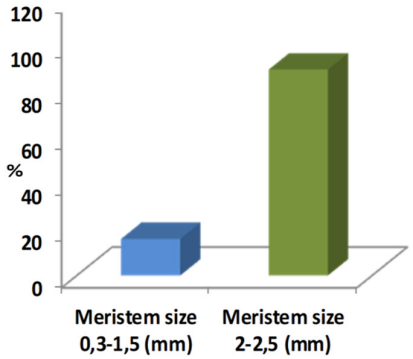

Figure 2. Percentage of garlic plantlets obtained in this study from the two apical meristem sizes: SM (0,3-1.5mm) and LM (2-2,5mm)

Assessment of virus remediation

Serologic analyses results showed that the size of the apical meristem greatly influenced viral elimination efficiency. In particular, the percentage of garlic virus remediation was of 8% in the plantlets obtained from LM apical meristems compared to 29% remediation reported for those obtained from SM meristems. From 2625 meristems excised, 2360 and 420 plantlets were obtained for the LM and SM groups, respectively. Regarding the virus –free plantlets, 190 plantlets for the LM and 122 plantlets for the SM were obtained (data not shown).

Results from this study showed that for a total of 80 plantlets infected by all viruses investigated and selected from LM (50) and SM (30) sizes, the remediation percentages were variable depending on virus and size. In particular, the percentage of remediation of viruses in the plantlets obtained from SM was 92.7% for the OYDV, 91.2% for LYSV, 57.4% for GCLV, 77.3% for SLV, 35.6% for the complex *Allexiviruses* (GarV-A, GarV-B and GarV-C). In the case of the LM the viral remediation percentages ranged between 13% for *Allexiviruses* investigated to 45% for OYDV. The virus-free plantlets obtained were 33% for SM and 8% for LM. The outcomes showed that OYDV, LYSV and SLV were easier to eradicate while, GCLV and the *Allexiviruses* (GarV-A, GarV-B and GarV-C) were the most persistent ones despite of the meristem size (Figure 3).

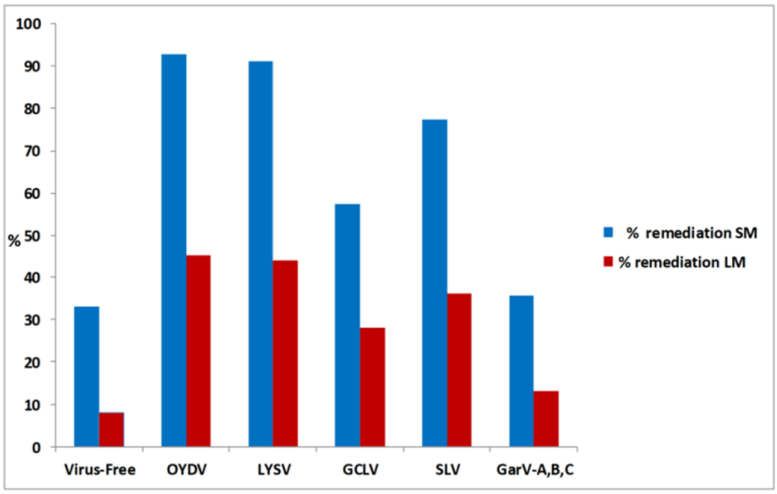

Figure 3. Viral remediation percentage of garlic plantlets obtained by micropropagation

Discussion

The immune-enzymatic and molecular investigations allowed us to identify the presence of viruses in the 105 accessions originated from Germany, Poland, Czech Republic, Italy and France. After serological investigations all garlic mother plants investigated resulted infected by 2 or more viruses. These data confirmed results of other studies which showed that the spread of viral infections is mostly caused by the accumulation of viral particles in the bulbs used for the obligatory vegetative propagation of the species (Fajardo *et al*., 2001; Paranno *et al*., 2012). Outcomes of the present study also showed that OYDV and LYSV were the most widespread viruses in the tested garlic accessions followed by GCLV and SLV. The major presence of OYDV and LYSV is probably due to their higher efficiency transmission via aphids in a non-persistent manner compared to GCLV and SLV even if they are transmitted in the same way (Van Dijk, 1993; Gadhave *et al*., 2020; Yang *et al*., 2021; Mansouri *et al*., 2021). The results of this study showed that the percentage of infection of the studied *Allexiviruses* was variable depending on country origin of the biological material. In fact, garlic accessions from Czech Republic, France and Italy were more infected by the investigated *Allexiviruses* (80%-

100%), while only 21% from Germany and 22% from Poland found to be infected confirming that the presence of *Allexiviruses* is different depending on the garlic origin (Chodorska *et al*., 2012; Chodorska *et al*., 2014; Bereda *et al*., 2017; Taglienti *et al*., 2018).

As expected, the RT-PCR outcomes revealed the presence of "false negatives" in the samples which resulted negative by ELISA. In fact, out of 105 analysed garlic accessions, only 1% of the samples tested by RT-PCR resulted free from OYDV in contrast with 4% revealed by ELISA. For LYSV, the percentage of virus-free plants was 4% in RT-PCR and 11.4% in ELISA, confirming the already well-known greater sensitivity (10-100 times) of PCR compared to ELISA as reported by other authors (Dovas *et al*., 2001; Meenakshi *et al*., 2006). In addition, the studies using molecular tools (Adams *et al*., 2005) and the Next Generation Sequencing (NGS) technology (Boonham *et al*., 2014; Ho *et al*., 2014; Kehoe *et al*., 2014; Mitiku *et al*., 2020) further expanded the existing knowledge about the considerable diversity of CP gene in plant viruses including those from garlic. This must be taken into account when applying ELISA as a diagnostic technique. In fact, the serologically detectable part of the protein corresponds to about one to two tenths of the capsid protein which, in turn, represents one or two tenths of the genome. Therefore, the serological analysis looks at the expression of a few hundred of the viral genomes while, with the molecular analysis, the whole viral genome can be detected (Boonham *et al*., 2014; Hadidi *et al*., 2016; Jones *et al*., 2017; Singh *et al*., 2020; Shahid *et al*., 2021; Prajapati *et al*., 2022). For this reason and also due to its greater sensitivity, molecular analysis is generally preferred to serological analysis.

Meristematic apexes *in vitro* culture is a commonly applied technique for virus-free garlic production. The tests performed in the current study, comparing two different dimensions of explanted meristem (0.3-1.5 mm and 2-2.5 mm), highlighted marked differences in the percentage of establishment. The apical meristems of larger dimensions and, therefore, with more leaf primordia, rooted with a much higher percentage than that obtained from smaller meristems equipped with a single leaf primordium. These outcomes are in agreement with those obtained by other authors who investigated meristem effects on garlic plant rooting and regeneration (Haque *et al*., 1999, Haque *et al*., 2003).

As reported in the literature (Luciani *et al*., 2006), the data obtained confirm the fundamental role of the explant size on obtaining virus-free material. The section of the apical meristem, therefore, directly influences the percentage of engraftment, and inversely, that of recovery. In order to produce healthy material, it is therefore necessary to identify the optimal size of the meristem to be explanted and to find a satisfactory compromise between the percentage of adaptation and recovery. Furthermore, the efficiency of viral elimination from the analysed *in vitro* material depends on the type of virus. For example, OYDV and LYSV were found to be more easily eradicated, although the latter was the most common virus in garlic. Conversely, our research outcomes showed that GCLV and the GarV-A, B, C viruses were found to be more difficult to eradicate.

Garlic is an important crop in Italy and worldwide. Therefore, obtaining virus-free propagation material is critical for increasing the overall yield and quality.

Future research is necessary, in order to obtain virus–free propagation material, assessing the optimal combination between the dimensions of the excised meristems eventually associated with other treatments (Matheus, 1991; Filho *et al*., 2006; Figliuolo and Mang, 2010; Panattoni *et al*., 2013; Ghaemizadeh *et al*., 2014; Pramesh *et al*., 2015) (e.g., thermotherapy and chemical treatments) suitable to obtain an elevated number of plantlets and virus elimination efficiency.

Conclusions

It was observed that garlic infection by viruses severely reduced the quantity and the quality of production due to modifications of the aerial part and bulbs aspect and taste of the final product. Four viruses namely, OYDV, LYSV, SLV and GCLV were identified by both ELISA and RT-PCR in all European countries investigated in single or mixed infections. The choice of meristem size greatly influenced virus elimination; smaller size (0,3-1,5 mm) allowed a better garlic viruses elimination efficiency than larger meristem size (2-2,5 mm). Instead, the regeneration of plants was better for the larger meristems then in case of the smaller ones. Furthermore, based on the outcomes of the present study is preferred to use smaller meristem size (0,3-1,5 mm) because it provides a higher virus elimination efficiency. In fact, it is better to obtain not as much of garlic plants but virus-free. Among all viruses investigated in this study the *Allexiviruses* (GarV-A, GarV-B and GarV-C) were the most difficult to eradicate compared to the *Potyviruses* and *Carlaviruses* studied.

Authors' Contributions

All authors contributed equally to this work.
All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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