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Simultaneous RT-PCR detection of 3 viruses infecting garlic (*Allium sativum* L.) crops in Ecuador and in vitro production of virus-free bulblets

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Andrés Eduardo Oleas Astudillo

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Dedico este trabajo a mis padres.

Ustedes son mi apoyo incondicional,

mi mejor ejemplo.

Les agradezco infinitamente

sus enseñanzas, amor y paciencia.

También te dedico este trabajo a ti,

Eres mi piel en invierno

Luz en mi camino

Sol en mi noche

Agua dulce en el mar

Mi vida, electrificas mi vida.

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ABSTRACT

Viral infection in garlic (*Allium sativum L.*) is a systemic disease that results in leaf striping, growth reduction and yield loss in the garlic production regions of Ecuador and the world. The disease is spread widely by seed bulbs due to the exclusive vegetative propagation of the crop, which causes virus particles to accumulate over generations. This is the first report of garlic infection by onion yellow dwarf virus (OYDV), leek yellow stripe virus (LYSV) and shallot latent virus (SLV) in Ecuador. Diagnosis was carried out using RT-PCR, a specific and sensitive method. Based on the individual detection of each virus, a protocol for multiple diagnosis was developed and tested. We used *in vitro* tissue culturing methods to propagate virus-free seed-bulblets from meristematic shoots. A regeneration ratio of 41% was achieved with a virus elimination rate of 2%. The age of developing-shoots at the excision time from the meristematic tissue determined if one or more virus species were partially or completely eliminated. This has been previously correlated to vascular tissue development in the sprouting shoot. SLV, a *Carlavirus*, was eliminated more frequently than the other two species of *Potyvirus* analyzed. Additional work should be carried out with the Ecuadorian varieties of garlic to improve virus-free seed-bulblet production rates.

RESUMEN

La infección viral en ajo (*Allium sativum L.*) es una enfermedad sistémica, causada por múltiples especies virales que resulta en clorosis, reducción de crecimiento y pérdidas de cosecha en las regiones productoras de ajo en el Ecuador y el mundo. La enfermedad es ampliamente esparcida por el bulbo-semilla debido a que la propagación vegetativa es la única opción para esta especie vegetal; esto causa la acumulación de partículas virales en cada generación. Este es el primer reporte en el Ecuador de infección en ajo causada por onion yellow dwarf virus (OYDV), leek yellow stripe virus (LYSV) y garlic latent virus (SLV). El diagnóstico se llevó a cabo utilizando RT-PCR, un método sensible y específico. Se desarrolló y experimentó un protocolo para el diagnóstico múltiple de las tres especies de virus mencionadas basándose en los protocolos de detección individual de cada virus. Un método de cultivo de tejidos *in vitro* se adaptó para la propagación de bulbillo-semilla libre de virus desde brotes meristemáticos. Se alcanzó una tasa de regeneración del 41%, con una tasa de eliminación viral del 2%. La edad del brote al momento de la escisión desde el tejido meristemático es un factor determinante en la eliminación, completa o parcial, de las especies virales que infectan el cultivo. Esto se ha correlacionado previamente a los tiempos de desarrollo del tejido vascular. SLV, un *Carlavirus*, fue eliminado con mayor frecuencia que las otras dos especies de *Potyvirus* analizadas. Este estudio pone la base para que se desarrolle investigación adicional para mejorar la producción de bulbillo-semilla libre de virus, específicamente para las variedades de ajo cultivadas localmente.

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1. INTRODUCTION

1.1 Garlic

Garlic (*Allium sativum* L.) is a worldwide-cultivated, economically-important plant species native to central Asia. It used for both culinary and medicinal purposes and has a history of human exploit of over 7000 years (Simonetti, 1990; Ensminger, 1994; Etoh & Simon, 2002). This perennial bulbous plant can withstand temperatures down to -29 ºC. It can grow up to 0.6 meters (m) in height and flowers rarely, since commercial cultivars are commonly sterile. When the plant does flower, it develops hermaphrodite flowers (Kamenetsky & Rabinowitch, 2002). The identification of the garlic wild progenitor is complex due to the aforementioned sterility, but several revisions have hinted at *Allium longicuspis* as the ancestor of *A. sativum*. The center of origin is thought to be located in central and southwestern Asia. The Mediterranean basin and the Caucasus region are considered secondary centers (Zohary & Hopf, 2000; Etoh & Simon, 2002)

There are two main subspecies; *A. sativum* var. *ophioscorodon* and *A. sativum* var. *sativum*; hardneck and softneck garlic respectively. The first subspecies encompasses varieties like Porcelain, Rocambole and Purple Stripe garlics, which develop scapes or “flower stalks”, whereas the latter subspecies includes well-known varieties such as Artichoke, Creole and Silverskin garlics, and is characterized by usually non-bolting plants. There is an intermediate group, generally considered hardneck garlics, which includes Asiatic and Turban garlics. These varieties have weakly bolting traits, meaning they develop scapes only under specific environmental conditions (Etoh & Simon, 2002; Anderson, 2013).

Scapes rarely give fertile flowers and usually produce topsets or bulbilbs which can be used for vegetative propagation of the plant, and grow to a normal bulb size in 1-2 years (WSU, 2013). Recently, there has been many studies focused on understanding the chemistry of garlic and its allegedly anti-fungal, anti-bacterial, anti-carcinogenic, anti-thrombotic, anti-inflammatory, anti-oxidant, lipoprotein balancing and blood pressure reducing properties, which seem to be a product of allicin and allicin-derived compounds (Srivastava, 1986; Silagy & Neil, 1994; Ankri & Mirelman, 1999; Banerjee et al., 2001; Keusgen, 2002; Elkayam et al., 2003; Bhagyalakshmi et al., 2005; Macpherson et al., 2005; Bautista et al., 2005). The biological effects of garlic seem to be enhanced in aged cloves and diminished in household and commercial preparations (Matsuura, 2008).

China encompasses more than 80% of the world garlic production with roughly 19 million tonnes grown every year. In contrast, Ecuador produces about 1500 tonnes each year (FAO, 2011; BCE, 2013). Garlic production is greatly diminished because of pests such as thrips (*Thrips tabaci*), mites (*Aceria tulipae*) and nematodes (*Ditylenchus dipsaci*), and phytopathogens such as fungi (*Sclerotium cepivorum, Fusarium* spp.) (MAG, 1986; INIAP, 2008). Studies indicate that at least twelve different viruses infect garlic naturally, although none has been reported in Ecuador, and it has been extensively shown that mixed viral infection reduces crop yields and quality. Losses of around 25-60% in bulb weight due to individual and multiple viral infections have been reported (Lot et al., 1998; Dovas, et al., 2001b; Conci et al., 2003; Cafrune et al., 2006; Lunello et al., 2007; Elnagar et al., 2009). Because garlic propagation is asexual, a lot of viral particles accumulate in plants and disease tends to get worse over generations (Robledo-Paz et al., 2000; Conci, et al., 2003).

1.2 Plant Viruses: A Global View

Viruses are metabolically inert, subcellular genetic particles with parasitic intracellular life cycles (Regenmortel, 2008). The majority of studied plant viruses are pathogenic to vascular plants of agricultural importance since they cause severe damage to crops by substantially reducing vigor, yield and product quality (Zaitlin & Palukaitis, 2000; Fuchs, 2008). The structure of a virus is given by its coat of proteins, which surround the viral genome. The majority of known plant viruses are rod-shaped (flexuous or rigid). Genome size determines the length of the virion, but dimensions usually range between 300–900 nm with diameters of 15–20 nm. About 65% of known plant viruses can have their genome translated as soon as it enters the cell (+ssRNA), but 10% have -ssRNA, meaning they must be transcribed to +ssRNA before they can be read by ribosomes (Hull, 2001).

Viral spread occurs by seeds, grafts, vectors (bacteria, fungi, nematodes and arthropods) or mechanically. Insects have proven to be especially efficient vectors for virus transmission. Malpractices such as monoculture or pesticide abuse tend to increase virus related problems as they usually result in massive population booms of aphids, major vectors for plant viruses (Blanc, 2008; Harries & Nelson, 2008; Cann, 2009). The plant cell wall must be broken in order to allow viral entry. Once inside cells, the virus needs to spread to other cells. Plant cell walls unavoidably have passages called plasmodesmata (PD) that allow cells to communicate with each other (Roberts, 2005). Transport through these channels is tightly regulated.

To allow the passage of virus particles or genomic nucleic acids, most plant viruses have evolved specialized movement proteins (MP) (Ghoshroy et al., 1997; Santa Cruz, 1999; Citovsky & Zambryski, 2005). Systemic spread of the virus is usually the case when the virus has compromised the vascular system by enlarging the plant PD to enter the sugar-transporting phloem sieve elements.

1.3 Viral Disease: Impact on Agriculture

Agricultural yield-losses due to plant virus-infections are estimated to range between 10% and 15% and represent more than $60 billion loses per year (Loebenstein, 2008). Transmission of viruses and the ensuing financial losses are more common in locations with evergreen vegetation throughout the year (Loebenstein, 2008). Virus related economic losses are mostly found in vegetative-propagated crops such as citrus, cassava, garlic, and potato, in comparison to seed-propagated ones. Viruses are usually unable to infect seeds; a defense mechanism thought to have occurred by evolution to ensure healthy progeny. In vegetative-propagated crops, sections of the adult plants are taken from infected plants for propagation, so the virus will invariably be present in the propagated material.

Garlic yield losses due to viral infection have been thoroughly reported. Experiments supporting the association between the presence of almost each one of the twelve garlic-infecting viruses to the reduction in bulb weight have been described (Conci et al., 2003; Cafrune et al., 2006; Elnagar et al., 2009).

Allexiviruses cause the least severe symptoms in crops and include garlic viruses (GarV) A, B, C, D, E, X and the garlic mite-borne filamentous virus (GarMbFV). Two aphid-borne carlaviruses, shallot latent virus also known as garlic latent virus (SLV/GarLV) and garlic common latent virus (GarCLV) also cause mild to non-existant symptoms, but seem to enhance the disease when present in mixed infections. Two potyviruses seem to be the most responsible of viral disease; a garlic strain of onion yellow dwarf virus (OYDV-G) and a specific strain of leek yellow stripe virus (LYSV-G) (Caciagli, 2008). A french report identified a potentially new garlic dwarf fijivirus (GDV) from the *Reoviridae* family as the causative agent of dwarfing and leaf thickening (Lot et al., 1994), although this virus has not been reported elsewhere.

1.4 The Case of Onion Yellow Dwarf Virus

OYDV belongs to the *Potyvirus* genus of the family *Potyviridae* which encompasses 30% of known plant viruses, many of which are of great agricultural significance (Chung et al., 2008). It is a +ssRNA non-enveloped aphid-borne virus and one of the major pathogens of onion and garlic appearing in viral complexes along with *Carlaviruses* and *Allexiviruses*. Its genome sequence has been determined (Soliman et al., 2012).

1.4.1 Host Range – Strains.

The virus has been isolated from naturally infected onion (*Allium cepa*), garlic (*A. sativum*) and *Narcissus pseudonarcissus* (Brierley & Smith, 1944, as cited in Bos, 1976), but its main natural hosts include shallot (*A. cepa* var. *ascalonicum*), *A. moly*, *A. scorodoprasum* and *A. vineale*.

It was also said to occur in Welsh onion (*A. fistulosum*) (Costa et al., 1971, as cited in Bos, 1976), although others found this species to be immune (Brierley & Smith, 1946, as cited in Bos, 1976). *Narcissus tazetta orientalis* and the true jonquil (*N. odorus regulosus*) showed symptoms after inoculation and the virus could be recovered from them (Henderson, 1935, as cited in Bos, 1976). Local lesions were obtained with some isolates in *Chenopodium amaranticolor* and *C. quinoa*. Virus isolates from onion produced no symptoms or only slight symptoms in *A. porrum* and the virus could only rarely be recovered (Bos, 1976).

Isolates of OYDV from different hosts have different levels of infectivity. Those from garlic and narcissus are less infective for onion and produce milder symptoms, thus probably representing different virus strains (Bos, 1976). A shallot virus isolate from the USA was easily transmitted to onion and infected all 27 onion cultivars tested, including Spanish-type cultivars immune to viral isolates from onion, garlic and narcissus (Brierley & Smith, 1946, as cited in Bos, 1976). Sumi et al. (2001) report two distinct set of primers for amplifying a region of the CP gene for garlic and onion isolates respectively, adding evidence to the possibility of viral strains related to host species.

1.4.2 Plant Symptoms and Effects on Agriculture.

Symptoms of OYDV occur first in leaves, emerging directly from infected bulbs as numerous, short, yellow streaked-spots in the base of such leaves. *A. cepa* (onion) and *A. cepa* var. *ascalonicum* (shallot) plants show yellow striping, leaf curling and plant stunting. As disease develops, OYDV-infected leaves become nearly completely chlorotic, partially flattened and crinkled, at which stage leaves abnormal in appearance wilt (Bos, 1976).

Narrow striped spots also occur along infected flower stems, which twist and crinkle; this lead to naming the disease onion yellow dwarf. Roots, fleshy leaves, floral parts and pollen all contain the virus at different concentrations. Basal portions of green leaves from plants 10-20 days after inoculation are known to be the best source of virus for mechanical transmission (Bos, 1976; Sutic et al., 1999; Conci et al., 2010). OYDV has been reported in virtually all the places in the world where onion, shallot or leek are cultivated. OYDV inhibits plant seed production by incapacitating the plants flowering processes. Vegetable yields of OYDV-infected plants in annual crops may be reduced by 17% to 60% depending on the virus-cultivar combinations, whereas potential seed yield losses may be reduced 50 to 75%. Virus-free stocks are re-infected within three to four growing seasons due to continuous influx from diseased plants growing nearby (Sutic et al., 1999; Majumder et al., 2008; Elnagar et al., 2009; Soliman et al., 2012).

1.4.3 Structure.

The OYDV elongated particles feature different reported lengths of 750 to 775 nanometers (nm) with diameters of 14 to l6 nm. The thermal inactivation point (TIP) of OYDV is 10 min at 60 to 65°C (some report 75 to 80°C). The dilution end-point (DEP) 10^{-2} to 10^{-4}. The longevity *in vitro* (LIV) is 2 to 3 days. OYDV has a single +ssRNA genome of 10.5 kb with a poly-adenine tail (Genbank accession number AB219833, AJ510223 or AB219834). The genome codes for a single polyprotein of 385.1 kDa that self-cleaves to produce all the proteins necessary for the virus life-cycle. Particles are flexuous filaments which can easily be detected in extracts of diseased onion or garlic leaves chopped in phosphotungstic acid, and often occur in aggregates (Bos, 1976; Chen et al., 2003; Takaki et al., 2006).

1.4.4 Transmission and Preventive Measures.

Some 60 aphid species transmit the virus non-persistently in nature. The most common vectors are; *Acyrthosiphon pisum*, *Aphis craccivora*, *Aphis fabae*, *Brachycaudus cardui*, *Hyalopterus pruni*, *Myzus ascalonicus*, *M. cerasi*, *M. persicae*, *Rhopalosiphum maidis* and *R. padi* (Bos, 1976). OYDV easily transmits in infective sap, but this mode of transmission is important only for experimental purposes. OYDV transmission through infected seed has never been reported although virus has been detected in pollen (Sutic et al., 1999). Production of healthy garlic bulbs mandates the prevention or reduction of infection by OYDV. During periodic inspections, all infected plants should be rogued and destroyed, particularly early in the season. To prevent sequential infections of bulb plants and seed bulbs for plant production adequate spatial isolation should be given. Shallot and onion, which represent a regular source of natural infection, should not be grown near garlic crops. Chemical control of aphids can be used to prevent infection in growing garlic bulbs and seed bulbs. All discarded garlic bulbs and commercial garlic residues should be destroyed because they serve as potential virus reservoirs after garlic harvest (Sutic et al., 1999).

1.5 The Case of Leek Yellow Stripe Virus

LYSV belongs to the *Potyvirus* genus of the family *Potyviridae* which encompasses 30% of known plant viruses, many of which are of great agricultural significance (Chung et al., 2008). It is a +ssRNA non-enveloped aphid-borne virus and one of the major pathogens infecting garlic in viral complexes along with *Carlaviruses* and *Allexiviruses*. Its genome sequence has been determined (Takaki et al., 2005)

1.5.1 Host Range – Strains.

The main natural host is leek (*A. porrum*), although the virus has been isolated from naturally infected onion (*A. cepa)* and shallot (*A. cepa* var. *ascalonicum*) when grown near severely infected leek (Graichen, 1978, as cited in Bos, 1981). Onion and shallot seem to be very resistant to the virus, but not immune (Bos et al., 1978, as cited in Bos, 1981). The virus has been experimentally shown to produce symptoms in *Chenopodium album, C. murale, C. foetidum, C. amaranticolor, C. quinoa, Celosia argenta* and *Nicotiana debneyi*. Welsh onion (*A. fistulosum*) is reported to be immune along with 23 other *Allium* spp. Six other *Allium* spp. have been reported to be susceptible and most were infected symptomlessly (Bos et al., 1978, as cited in Bos, 1981; Graichen, 1978, as cited in Bos, 1981).

Wei et al. (2006) reported two distinct strains of LYSV co-infecting the same garlic plant based on sequence differences of approximately 15% for a fragment which included the Nib gene (RNA dependent RNA polymerase or RdRp), the CP gene and 3'UTR regions. It is important to stress that restricted host ranges are a common feature of potyviruses (Van Dijk, 1993a) and that these ranges, along with symptom induction and aphid transmissibility can be altered by point mutations in the viral genome (Atreya et al., 1991; Atreya et al., 1992; Riechmann et al., 1992).

1.5.2 Plant Symptoms and Effects on Agriculture.

Symptoms rarely present during summer months, but become increasingly apparent in fall months. Darker-colored leek varieties show some degree of resistance to LYSV (Sullivan & Robinson, 2012), apparently thanks to a thicker wax layer (Van Dijk, 1993a).

LYSV causes dwarfing of the entire plant and yellow striping on garlic leaves, especially on the distal parts (Sullivan & Robinson, 2012). *Allium* crops infected with LYSV are more susceptible to weather conditions like frost and do not keep well post-harvest (Bos et al., 1978). Garlic yield loses vary depending on each cultivar, but reduction of bulb weight can range from 20 to 60%. Co-infection with other viruses, especially OYDV and SLV, can aggravate symptoms and decrease bulb weight up to 80% (Conci, 1997; Lot et al., 1998; Lunello et al., 2007). Epidermal cells contain two cytoplasmic inclusion bodies on average which can be confused with the cell's nucleus (Bos, 1981). In ultrathin sections, pinwheel, tubular and laminar viral inclusions can be observed, these are characteristic of potyviruses (Conci, 1997; Lunello et al., 2002).

1.5.3 Structure.

LYSV virions consist of flexuous particles with a modal length of 820 nm that tend to aggregate end-to-end in sap (Verhoyen & Horvat, 1973, as cited in Bos, 1981). TIP is 10 min at 50 to 60°C, DEP is 10^{-2} to 10^{-3} and LIV is 3 to 4 days (Graichen, 1978, as cited in Bos, 1981). In unpublished data, Bos states the virus can be conserved in dried leaves treated with $CaCl_2$ for at least 9 years. LYSV has a single +ssRNA genome of 10.2 kb with a poly-adenine tail (Genbank accession number JX429967, JQ899450, HQ258895 or JX429965) which codes for a polyprotein of 364.4 kDa that self-cleaves to produce various proteins needed for the virus life-cycle.

1.5.4 Transmission and Preventive Measures.

The virus is transmitted non-persistently by aphids including; *M. persicae* (green peach aphid), *A. fabae* (black bean aphid), *R. maidis, R. padi, Schizaphis graminum, A. gossypii, A. nerii, Uroleucon sonchi* and *Hyperomyzus carduellinus* (Lunello, 2002; Sulivan & Robinson, 2012). The virus cannot be transmitted by seed produced from virus-infected plants (Bos, 1981), although this is only relevant in leek as garlic propagates in an agamic fashion and thus bulbils accumulate virus particles each generation (Robledo-Paz et al., 2000; Conci, et al., 2003).

Production of healthy garlic bulbs mandates the prevention or reduction of infection by LYSV. During periodic inspections, all infected plants should be rogued and destroyed, particularly early in the season. To prevent sequential infections of bulb plants and seed bulbs for plant production adequate spatial isolation should be given (Sutic et al., 1999). Leek, which represents a regular source of natural infection, should not be grown near garlic crops. Chemical control of aphids can be used to prevent infection in growing garlic bulbs and seed bulbs. All discarded garlic bulbs and commercial garlic residues should be destroyed because they serve as potential virus reservoirs after garlic harvest (Sutic et al., 1999).

1.6 The Case of Shallot Latent Virus

SLV belongs to the *Carlavirus* genus of the family *Betaflexiviridae*, which is characterized by having triple gene block (TGB) proteins. These proteins facilitate cell-to-cell and long-distance movements (Swiss Institute of Bioinformatics, 2013).

It is a +ssRNA non-enveloped aphid-borne virus and one of the major viral pathogens of garlic, appearing in viral complexes along with Potyviruses and Allexiviruses. Its genome sequence has been determined (Wylie et al., 2012).

1.6.1 Host Range – Strains.

The virus has a limited host range, being capable of infecting less than 3 families. Leek (*A. porrum*), onion (*A. cepa*) and garlic (*A. sativum*) are natural hosts with the virus being almost omnipresent in shallot (*A. cepa* var. *ascalonicum*). *A. fistolosum* and *A. jailae* have been infected under experimental conditions along with *C. album, C. amaranticolor* and *C. quinoa*; this latter group serves as a good diagnostic species. *A. neapolitanum, A. schoenoprasum, Freesia* spp., *Hyacinthus* spp., lily *(Lilium formosanum), Narcissus*, some *Tulipa* cultivars, and 15 other plant species tested appear to be immune (Bos, 1982; Brunt et al., 1996).

SLV receives its name after being first isolated from shallot (Bos et al., 1978), but it was also isolated separately in Japan and named garlic latent virus (Lee et al., 1979, as cited in van Dijk, 1993b); the consensus now is that these two represant strains of the same virus (van Dijk, 1993b; Conci, 1997; Tsuneyoshi et al., 1998a). Based on aminoacid sequence differences of the CP core region, Chen et al. (2001) described 3 strains of SLV in accord to International Comittee on the Taxonomy of Viruses (ICTV) criteria. ICTV states that less than 68% amino acid identity demarks a different carlavirus species while strains of the same virus have 75-90% of identical aminoacid sequence (Van Regenmortel et al., 2000).

Tsuneyoshi et al. (1998a) found characteristic and common variations in CP sequences suggesting the possible presences of geographical variants, and found no apparent sequence variations related to host plant species among 6 infected *Allium* crops.

1.6.2 Plant Symptoms and Effects on Agriculture.

SLV infects shallot, onion, garlic, *A. fistolosum* and *A. jailae* symptomlessly. Infection causes mild chlorotic streaking in leek when occurring alone. Severe chlorotic streaking and even plant death have been reported in some cultivars of leek when in complex with LYSV (Paludan, 1980, as cited in Bos, 1982). Light microscopy is not useful because SLV does not form inclusion bodies, although electron microscopy reveals aggregates of the virus, especially around cell membranes. In *C. amaranticolor* and *C. quinoa,* small necrotic (older leaves) or chlorotic (younger leaves) local lesions develop from 6 to 10 days after inoculation. These lesions become green rings surrounding necrotic tissue when inoculated leaves turn yellow (Bos, 1982).

There are no reports on garlic yield reduction from a SLV-only infection, but it is clear that this virus aggravates symptoms and economic losses (reduction in bulb weight) when in conjunction with potyviruses such as LYSV and OYDV (Conci, 1997; Lot et al., 1998; Lunello et al., 2007), thus this virus plays an important synergistic role in garlic viral disease.

1.6.3 Structure.

Virus particles consist of straight or slightly curved filaments with a modal length of 650 nm that tend to aggregate end-to-end in sap and are likely to appear as dimers, being less flexuous than LYSV and OYDV (Bos, 1982). TIP is 10 min at 80°C, DEP is 10^{-4} to 10^{-5} and longevity in crude sap is 8 to 11 days (Bos, 1982). SLV has a single +ssRNA genome of 8.4 kb with a poly-adenine tail (Genbank accession number HQ258896, JF32 0811, JQ899443or JX429965). The genome codes for a RdRp and has a triple gene block (TGB); a set of three peptides whose expression is regulated by the same sub-genomic promoter and all of which are involved in cell-to-cell and long-distance movements (Swiss Institute of Bioinformatics, 2013).

1.6.4 Transmission and Preventive Measures.

The virus does not replicate in the host and stays localized in the piercing stylet of the aphid. When the vector feeds, this specialized apparatus breaks the plant tissue and deposits the virus (Sullivan & Robinson, 2012). Some propose that aphid vectors infect the growing plants too late into the growing season to cause economic losses in that same season (Davis, 2008). *M. ascalonicus* and *A. fabae* are known vectors, but not *M. persicae* (Bos, 1982). Production of healthy garlic bulbs mandates the prevention or reduction of infection by SLV. Periodic inspections should be carried out using ELISA as the virus infects garlic symptomlessly. Shallot, which represents a regular source of natural infection, should not be grown near garlic crops.

Chemical control of aphids can be used to prevent infection in growing garlic bulbs and seed bulbs. All discarded garlic bulbs and commercial garlic residues should be destroyed because they serve as potential virus reservoirs after garlic harvest (Sutic et al., 1999).

1.7 Viral Diagnosis

Accurate techniques for routine detection of plant viruses tend to be limited. This fact highlights the need of low cost methods for diagnosis that maintain appropriate sensitivity and specificity. Methodologies that permit simultaneous detection of multiple plant viruses (multiplexing) reduce the number of assays required, reagent usage, time spent, and consequently, the cost. Multiplex polymerase chain reaction (PCR), polyvalent PCR, real-time PCR (qPCR) and micro-array technologies allow simultaneous detection of multiple plant viruses in a single assay.

The increased sensitivity achieved with some techniques, such as real-time PCR, may require more time and money in comparison to less sensitive protocols. The decrease in false-negatives is a benefit that outweighs the cost, as the risk of having a shipment returned to an exporter because of viral infection is greatly reduced (James et al., 2006). Multiplexing techniques can target various taxonomic levels and thus have the capacity for simultaneous broad-spectrum and specific identification. Polyvalent PCR using broad-spectrum primers have the potential to detect unknown or uncharacterized viruses, improving the ability to monitor and successfully control these pathogens.

Techniques such as micro-array analysis offer the potential for development of a single biochip that may facilitate detection of all viruses affecting a particular crop. Currently, the Enzyme Linked Immunosorbent Assay (ELISA) is the main diagnostic method for large-scale routine detection of viruses in onion and garlic (Dovas et al., 2001a; Salomon, 2002; James et al., 2006; Meenakshi et al., 2006).

However, homogeneous preparation of viral particles for specific antibody production is complicated due to mixed infection of several viruses in garlic, onion and other *Allium* crops. Moreover, the high variability in the epitope coding regions of the CP gene results in the occurrence of serologically different strains of the same species of virus. ELISA, therefore, is not a preferable method of detection. Protocols like IC-PCR, RT-PCR with plant tissue extracts, and RT-PCR with total RNA, have proved to be 10^2-10^4 times more sensitive than double-antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA) (Dovas et al., 2001a; Salomon, 2002; James et al., 2006; Meenakshi et al., 2006).

1.8 Generation of Virus Free Plantlets

Meristem tip culture is a diverse and well-established method for eliminating viruses by regenerating virus-free plantlets. The first approaches at the subject were made in the 1970-1980s and focused on generating callus from the meristem tips of young leaves, inducing shoot multiplication and then bulb formation (Bhojwani et al., 1982; Peña-Iglesias & Ayuso, 1983; Bertaccini et al., 1986; Walkey et al., 1987; Walkey & Antill, 1989).

These procedures have the limitation of producing only 1 callus per bulb and so other researchers looked for alternative sources of meristematic tissue. Scape tips and root tips proved to work and took advantage of a usually discarded tissue (Ma et al., 1994; Myers & Simon, 1998; Robledo-Paz et al., 2000). These techniques are labor-intensive and time-consuming with low virus-free propagation rates (Bhojwani et al., 1982; Walkey, 1987). Many have reportedly improved the efficiency of propagation (Havránek & Novák 1973; Kehr & Schaeffer 1976; Abo El-Nil 1977; Nagakubo et al., 1993). Nevertheless, there are inescapable flaws that limit the practicality of these methods; the need for long-term cultivation, relatively low propagation rates, and the necessity of mastering skillful techniques.

The research conducted by Nagasawa & Finer, and Xue et al. (1988; 1991) hinted at the usefulness of the basal part of the garlic clove as a potent explant. This fact was further exploited by Ayabe and Sumi (1998). They developed an efficient tissue culture technique suitable for micropropagation and the reported stem-disc dome culture (SD-Dome Culture) technique, capable of producing virus-free garlic bulblets (Ayabe & Sumi, 2001). This technique has higher virus-free propagation rates and requires less amount of time required to go from bulb to virus-free bulblet, as it does not have a callus phase. This method is also hormone-free (Ayabe & Sumi, 2001).

Thermotherapy has been found to aid in virus elimination. Viral proliferation seems to be reduced or completely stopped at temperatures above 38ºC. At this temperature plant cells are still capable of slow division, allowing for the generation of virus free tissues (Salomon, 2002).

This fact improves the chance of regenerating virus-free propagules but tends to reduce the viability of the explants (Ucman et al, 1998). It has also been reported that thermotherapy inhibits OYDV eradication (Shiboleth et al., 2001) which seriously reduces the applicability of this method. Each report should be carefully analyzed individually as there is not a standard method for the application of thermotherapy.

Chemotherapy is another option for virus elimination and it consists in using chemicals, mainly ribavirin, that interfere with nucleic acid replication. This method has been described by Salomon (2002) as achieving little success, but his argument seems to be based on only 1 reference and somewhat biased. Ramirez-Malagón et al. (2006) report using "embryo axes" cultured in Gamberg's B-5 medium supplemented with ribavirin [50 mg L^{-1}] and having a 100% survival rate (out of 5000 explants) and a 27-35% virus-elimination rate depending on the cultivar. It is important to highlight that the efficiency of virus-free plant propagations has been shown to be cultivar-dependent (Koch et al., 1995; Verbeek et al., 1995; Ucman et al., 1998; Shiboleth et al., 2001).

Soliman et al. (2012) report using basal plates as explants and applying ribavirin on media for shoots subcultured from each explant. They describe a decreasing survival rate with increasing ribavirin concentration and an increased virus-elimination rate as well. Electrotherapy has been recommended for the cleansing of a variety of viruses in different crops, and Soliman et al. (2012) claim it works best when combined with chemotherapy, although they advise against each of those treatments independently for OYDV eradication.

2. OBJECTIVES

2.1 General

The aim of this study was to produce virus-free garlic seed-bulblets through tissue culture and develop a single, sensitive, specific and efficient method for the detection of OYDV, LYSV and SLV.

2.2 Specific

- To standardize individual and triplex RT-PCR capable of detecting OYDV, LYSV and SLV.
- To establish a tissue culture technique for the micropropagation of garlic.
- To standardize the culture of dome shaped meristematic tissue for the generation of virus-free garlic seed-bulblets.

3. JUSTIFICATION

In Ecuador, garlic is cultivated exclusively in the highlands region; being Azuay, Cañar, Carchi, Chimborazo, Loja, Pichincha and Tungurahua the provinces that encompass the majority of the national production. Garlic *blanco* and *rosado* are the most commonly grown varieties, and their production is greatly diminished because of pests such as thrips (*Thrips tabaci*), mites (*Aceria tulipae*) and nematodes (*Ditylenchus dipsaci*), and phytopathogens such as fungi (*Sclerotium cepivorum, Fusarium* spp.) (MAG, 1986; INIAP, 2008). Studies indicate that at least twelve different viruses infect garlic naturally, although none has been reported in Ecuador, and it has been extensively shown that mixed viral infection reduces crop yields and quality. Losses of around 25-60% in bulb weight due to individual and multiple viral infections have been reported (Lot et al., 1998; Dovas, et al., 2001b; Conci et al., 2003; Cafrune et al., 2006; Lunello et al., 2007; Elnagar et al., 2009). Because garlic propagation is asexual, a lot of viral particles accumulate in plants and disease tends to get worse over generations (Robledo-Paz et al., 2000; Conci, et al., 2003).

This results in an inevitable economic loss for the country as national demand requires importation of garlic to meet the need of this product. For 2008, more than 1400 tonnes of garlic were produced in Ecuador. Still, around 432 tonnes had to be imported, mainly from China, to satisfy domestic demand. This represented a expenditure of CIF (cost, insurance, freight) $229 350 which could be avoided in the future if national production of world-quality bulbs increases (MAGAP, 2011; BCE, 2013). Production could be enhanced to the point where the country is able to export a high-quality virus-free product.

Because garlic propagation is asexual, several virions accumulate in bulb-seeds and viral disease tends to get worse over generations (Robledo-Paz et al., 2000; Conci, et al., 2003). There appears to be no virus-free propagation material in the country at the moment and thus we look to reproduce a previously reported *in vitro* tissue culture technique that propagates plants from meristem, apparently the only virus-free tissue in the plant (Ayabe & Sumi, 2001). To diagnose the garlic plants, we have chosen one of the most sensitive techniques available, namely RT-PCR (Dovas, et al., 2001; Shiboleth et al., 2001; Salomon, 2002). This molecular technique will ensure a sensitive and specific diagnosis of the viruses affecting garlic.

This study seeks to contribute a solution to the lack of virus-free plant material in Ecuador and thus increase national garlic production by providing a method for reducing yield-losses associated with viral infection. Furthermore, we also look to develop a highly efficient, sensitive and specific method which may allow the certification of virus-free plant material, thus enabling the possibility of international commerce.

4. FIELD OF STUDY

Garlic (*Allium sativum* L.) cultivated at USFQ experimental farm in Tumbaco, Ecuador (0° 13' 12"S, 78° 24' 0"W) was used in all the experiments. *In vitro* tissue culture of garlic as well as molecular diagnosis and analysis was carried out at Laboratorio de Biotecnología Vegetal in Universidad San Francisco de Quito (USFQ). DNA sequencing was done at Functional Biosciences labs in Wisconsin, US.

5. MATERIALS

5.1 Viral Diagnosis

5.1.1 Total RNA Extraction.

- Garlic leaves and cloves from USFQ experimental farm in Tumbaco
- 0.1% diethyl pyrocarbonate (DEPC) Sigma Aldrich™
- Trizol Reagent Invitrogen™
- Chloroform Merck™
- Isopropyl alcohol Merck™
- Citrate buffer (0.8 M monosodium citrate Merck™, 1.3M NaCl Fisher Scientific™, DEPC-treated H_2O Invitrogen™)
- 75% ethanol (EtOH) J.T. Baker™
- DEPC-treated H_2O Invitrogen™
- Eppendorf™ tubes washed with DEPC
- Mortars washed with DEPC and sterilized in an autoclave
- Tweezers and scalpel washed with DEPC and sterilized in an autoclave
- 5415R Eppendorf™ microcentrifuge
- Analytic scale LA230S Sartorius™
- Spectrophotometer NanoDrop 1000 Thermo Scientific™
- SPSS software (SPSS Inc., 2008)
- Microsoft Excel 2010

5.1.2 First-Strand cDNA Synthesis.

- DEPC-treated H_2O Invitrogen™
- dNTP Mix 10 µM Invitrogen™
- Not I-d(T_{18}) primer Invitrogen™ (Appendix A)
- SuperScript III Reverse Transcriptase Invitrogen™
- 5X First Strand Buffer Invitrogen™
- DTT (Dithiothreitol) 0.1 M Invitrogen™
- Garlic total RNA
- 5415D Eppendorf™ microcentrifuge
- TPersonal Biometra™ Thermocycler

5.1.3 cDNA Amplification.

- DEPC-treated H2O Invitrogen™
- dNTP Mix 10 µM Invitrogen™
- O3, S and L reverse and forward primers Invitrogen™ (Appendix A)
- Recombinant Taq DNA Polymerase Invitrogen™
- 10X PCR Buffer minus Mg Invitrogen™
- $MgCl_2$ 50 mM Invitrogen™
- cDNA retrotranscribed from Garlic total RNA
- TPersonal Biometra™ Thermocycler

5.1.4 Gel Band Visualization, Extraction and Reamplification.

- Agarose LE Axygen™
- 100 bp ladder Axygen™
- 100 bp TrackIt DNA ladder Invitrogen™
- Tris Base Invitrogen™
- Boric acid Amp™
- EDTA Invitrogen™
- Electrophoresis chamber Enduro Gel XL Labnet™
- Power Source EPS-300 II C.B.S Scientific Company™
- SYBR Safe Invitrogen™
- Blue Juice Invitrogen™
- Gel-Doc XR molecular imager BioRad™
- DEPC-treated H_2O Invitrogen™
- dNTP Mix 10 µM Invitrogen™
- O3, S and L reverse and forward primers Invitrogen™ (Appendix A)
- Recombinant Taq DNA Polymerase Invitrogen™
- 10X PCR Buffer minus Mg Invitrogen™
- $MgCl_2$ 50 mM Invitrogen™
- Agarose extracted DNA
- TPersonal BiometraTM Thermocycler
- PureLink® Quick Gel Extraction Kit Invitrogen™
- Quantity One® analysis software BioRad™

5.2 Sequencing and Analysis.

- Molecular Evolutionary Genetics Analysis (MEGA) 5.1 software (Tamura et al., 2011)
- Mr Bayes 3 (Ronquist & Huelsenbeck, 2003)
- TOPALi v2 (Milne et al., 2008)
- Tracer v1.4 (Rambaut & Drummond, 2007)
- Basic local alignment search tool (BLAST) algorithm (NCBI) (Altschul et al., 1997)
- Geneious R7 (Biomatters, 2013)
- ABI 3730xl DNA Sequencer at Functional BioSciences lab

5.3 Tissue Culture

5.3.1 Stem Disc Culture.

- Garlic cloves from USFQ experimental farm in Tumbaco
- 75% potable ethanol (EtOH) solution J.T. Baker™
- Distilled sterile H_2O
- LS medium (Appendix B), sucrose 30 g L^{-1}, Agar Sigma-Aldrich™, pH 5.6
- pH Meter Orion Star A111 Thermo Scientific™
- Laminar flow hood HERA Guard HPH15 Thermo Scientific™
- Stereo microscope Reichert-Komp™ 12,5X
- Benomyl®
- Autoclave Trident EA632

5.3.2 Shoot Extraction.

- Laminar flow hood HERA Guard HPH15 Thermo Scientific™
- LS medium (Appendix B), sucrose 30 g L^{-1}, Agar Sigma-Aldrich™, pH 5.6

5.3.3 Transference to soil.

- 250cc plant pots
- Black páramo soil
- Vermiculite
- Organic compost from USFQ's experimental farm
- Urea Ecuaquímica™
- Potassium Chloride Ecuaquímica™
- Vitafol®

6. METHODOLOGY

6.1 Viral Diagnosis

6.1.1 Total RNA Extraction.

Total RNA was extracted from 60-200 mg of phenotypically-ill garlic cloves and leaves, from onion leaves and from 10-120 mg of garlic leaves from plants that underwent tissue culturing. The phenol-chloroform Trizol protocol was used according to the Invitrogen's instructions (Appendix C) with the following modifications:

1) Homogenization was carried out using a mortar and pestle to crush plant tissues after they were frozen solid with liquid nitrogen, the amount of tissue used for homogenization was much higher than the amount actually used for the rest of the extraction.
2) Before the RNA precipitation step 300 μL of citrate buffer was added to each sample.
3) A centrifugation step of 20 minutes at 12000 rpm 4ºC was done after resuspending the RNA to eliminate undissolved residues. RNA was quantified using NanoDrop1000 (Themo Scientific) and visualized after electrophoresis in 1.5% agarose gels for 45 minutes at 80 volts.

A possible bivariate correlation between the amount of tissue used for the extraction and the organic purity, protein purity and amount of RNA concentration obtained was tested with SPSS v17 using Pearson's R, Kendall's Tau-b and Spearman's Rho correlation coefficients.

6.1.2 First-Strand cDNA Synthesis.

0,1 µL SuperScript™ III Reverse Transcriptase (Invitrogen, USA) was used in the reaction mixture (Appendix D) in a final volume of 20 µL. Initially the PCR specific reverse primer of each virus was used to separately reverse transcribe each viral target sequence, but Not I-d$(T)_{18}$ was eventually adopted as a universal reverse transcription primer thanks to its poly-T domain; so a single reverse transcription reaction could be done for the 3 viruses.

6.1.3 cDNA Amplification.

Three pairs of primers were used independently and in a triplex PCR; primer O3 amplified the 3' region of the CP gene and part of the 3' untranslated region (UTR) of OYDV, primer S amplified the 3' region of the CP gene of SLV and primer L amplified a part of the 3' UTR of LYSV (Fig 1). Gradient PCR was done for every reaction to determine the best annealing temperature. 1 µL of cDNA and 0,2 µL of recombinant Taq DNA Polymerase (Invitrogen, USA) were used in a final reaction volume of 20 µL. Three additional pairs of primers were tested (O1, O2 and Pot), 2 for OYDV and 1 for potyviruses OYDV and LYSV. Reaction mixtures, thermocycler programs and primer sequences are described in detail in Appendix A.

6.1.4 Gel Band Visualization, Extraction and Re-amplification.

Triplex RT-PCR products were visualized after 120 minutes of electrophoresis at 80 volts in a 3% agarose gel with SybrSafe [7µL/100mL] and photographed under UV light with the Bio-Rad XR photo-documentation system.

Individual amplifications were visualized after 45 minutes of electrophoresis at 80 volts in a 1.5% agarose gel. Amplicons were recovered from the gel with the PureLink Quick Gel Extraction kit (Invitrogen) and reamplified for sequencing. The re-amplification was done with recovered DNA under the same conditions for individual amplifications (Appendix A).

6.2 Sequencing and Analysis

Amplified and reamplified products were sequenced at Functional BioScience laboratories in Wisconsin, USA using an ABI Sequencer. Consensus sequences were obtained using MEGA version 5 (Tamura et al., 2011). The basic local alignment search tool (BLAST) algorithm from the National Center for Biotechnology Information (NCBI) was used to find closely related sequences reported in GenBank (Altschul et al., 1997). Only sequences with query coverage of 99% or more were considered for maximum identity comparison, unless specifically stated otherwise.

Sequence alignment was done using the progressive alignment tool Clustal W (Thompson et al., 1994). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (Tamura et al., 2011), TOPALi version 2.5 (Milne et al., 2008), Geneious version 7 (Biomatters, 2013) and Mr Bayes version 3 (Ronquist & Huelsenbeck, 2003). Phylogenetic trees were generated after selecting the nucleotide substitution model (NSM) that best fitted the data according to the Bayesian Information Criterion (BIC) and the Akaike Information Criterion (AIC) value generated for each model by TOPALi and MEGA (Posada & Crandall, 2001).

Starting phylogenetic trees for maximum likelihood analysis were constructed using the evolutionary distance matrix method Neighbor Joining integrated in MEGA 5.1 (Tamura, et al., 2011) software. For Bayesian analysis, flat dirichlet distributions (Mr Bayes defaults) were used for the priors, two independent Markov chain Monte Carlo runs were performed on each analysis with 4 chains (1 cold and 3 hot) each, the analysis ran for 10^6 generations and 33% of the samples were discarded as burn-in.

Each analysis was repeated at least three times to account for the initial random tree used. Convergence of the runs was determined by: achieving a standard deviation of split frequencies value of less than 0.05, each parameter having a potential scale reduction factor (PSFR) value near 1 and by analyzing the samples in Tracer version 1.5 (Rambaut & Drummond, 2007); checking for adequate mixing and effective sample size (ESS).

6.3 Tissue Culture

6.3.1 Stem Disc Culture.

Garlic cloves were pre-treated at 4ºC for at least 4 weeks to enhance bulb formation rates (Ayabe & Sumi, 1998). Cloves were first peeled and washed for at least 1 min with tap water, next they were cut transverlesy and half containing the stem-disc was sterilized in 75% EtOH for another 5 min and finally washed with sterile distilled water. Initially 3% sodium hypochlorite was used after EtOH for 10 min, but skipping this sterilization step did not result in an increase in contamination rates; consequently this step was eliminated.

Residual storage and foliage leaves were further cut using a stereo microscope and a 1-2 mm thick disc was extracted (Fig 2), cut in half and placed in petri dishes containing hormone-free Linsmaier-Skoog (LS) medium (Linsmaier & Skoog, 1965) (Appendix B). Four stem discs were cultivated per petri dish. Murashige-Skoog (MS) medium was also used for the starting assays along with fungicide (Benomyl) at a concentration of 3 g L^{-1}. Dishes were incubated at an average temperature of 22ºC under a 16 hours light – 8 hours dark photoperiod with cool white fluorescent illumination, 40 µmol m^{-2} s^{-1}.

6.3.2 Shoot Extraction.

0.5-15 cm shoots were removed from the surface of the stem disc with little pressure 2-8 weeks after the stem disc was cultured and planted on hormone-free LS medium in test tubes. Tubes were incubated at an average temperature of 22ºC under a 16 hours light – 8 hours dark photoperiod with cool white fluorescent illumination 40 µmol m^{-2} s^{-1}.

6.3.3 Direct Bulb Formation.

Sprouting shoots were left on the stem disc for 6 to 12 weeks. Once the shoots developed roots, they were washed with tap water and planted in a pot, stem disc remnants were discarded. Bulblets were stored at 4 ºC for at least 1 month to simulate vernalization and then planted in soil.

6.3.4 Transference to soil.

After 6-8 weeks of the shoot extraction process, shoots that developed a strong root system and had leaves at least 8 cm high were planted on 250cc pots with a substrate mix of: 2 parts black páramo soil, 1 part compost from the experimental farm and 1 part vermiculite. Solid fertilizers containing urea and potassium chloride were used along with foliar fertilizer (Vitafol®) to strengthen the plantlets and maximize bulblet size.

All pots were kept in a greenhouse. Plantlets were put in pots directly from the test tubes, i.e. no intermediate acclimatization steps that lowered the relative humidity progressively were carried out. Rooted shoots and vernalized bulblets were planted in the same substrate mix as extracted shoots.

6.4 Virus Elimination Diagnosis

RNA was extracted from leaves or cloves before culturing the stem disc and during the transference to soil. Plantlets obtained from shoots extracted at different times and of different lengths, and from the direct bulb formation process were diagnosed. Shoot regeneration, bulb formation and virus elimination rates were evaluated. Stem discs were cultured as described in the stem disc culture section. Each diagnosis was done by duplicate, from the RNA extraction step. For SLV, negative samples were re-confirmed by single PCR, i.e. by amplification with the S primer only.

7. RESULTS

7.1 Viral Diagnosis Standardized Protocol

Protocols for individual and multiple diagnosis of LYSV, OYDV and SLV were standardized (Fig 3). Although SLV bands detected in triplex PCR were somewhat faint, every SLV diagnosis result was confirmed by individual amplification. Triplex RT-PCR required different reagent concentrations and temperature profiles than single PCR for specific amplification (Appendix A). SLV, LYSV and OYDV were unambiguously detected down to RNA quantities of 55 ng from less than 10mg of plant tissue. Potyviruses could be detected down to RNA quantities of 30 ng, however SLV showed no amplification with this amount of RNA.

7.1.1 Total RNA Extraction.

RNA was obtained from garlic leaves and cloves consistently with the phenol-chloroform Trizol reagent extraction protocol (Appendix C). On average samples had a 1.8 260/280 and 0.7 260/230 ratio of absorbance. RNA concentrations ranged from 30-3480 ng μL^{-1}. Samples across the whole range of concentration were successfully reverse transcribed and amplified. Total RNA samples were visualized after 45 minutes of electrophoresis at 80 volts in a 1.5% agarose gel. The amount of tissue used during the homogenization step influenced the success of the extraction; this is further explained in the discussion section.

RNA quantity and protein quality estimation performed by NanoDrop 1000 was inaccurate since most spectrophotographs showed high absorbances at wavelenghts of 230 nm and 270 nm, indicative of contamination with phenolate, thiocyanates and other organic compounds, and phenol respectively. A statistically-significant, low positive-correlation was found between the amount of tissue used for the extraction (10-120 mg range) and the protein purity, organic purity and RNA concentration obtained (Table 1) for the 172 RNA samples extracted, the scatter plots can be seen in Appendix E.

7.1.2 First-Strand cDNA Synthesis.

The result of reverse transcription reactions could only be assessed by the amplification of the cDNA produced. Positive amplifications were obtained from reverse transcription products obtained with Not I-d(T_{18}) and with the reverse primer of each virus.

7.1.3 cDNA Amplification.

Individual and triplex amplifications with primers L, O3 and S yielded the expected amplification products (Fig 3). Annealing temperatures, as determined by gradient-PCR, were 60ºC, 63ºC and 53ºC for primers L, O3 and S respectively. Triplex PCR optimal annealing temperature was 57ºC. Non-specific bands were eliminated by a combination of: increased annealing temperature, shorter time during the annealing step and increased buffer and $MgCl_2$ concentration.

The temperature profile for triplex PCR was that of the O3 primer with the annealing time of the S primer (Appendix F). Amplification yielded specific bands most of the time, but in some cases extra faint bands were detected for SLV. Primer O1 showed no amplification in garlic or onion in an annealing temperature range of 44-52ºC, its reported annealing temperature is 48ºC (Majumder et al., 2008). Primer O2 produced 601 bp amplicons in shallot, but not in garlic (Fig 4). Primer Pot ampplified many bands, but none correponding to the reported amplicon size (Tsuneyoshi et al., 1998b).

7.1.4 Gel Band Visualization, Extraction and Reamplification.

Amplicon size was estimated by visual comparison with the molecular ladder and by using the Quantity One® analysis software; LYSV, OYDV and SLV bands were around 191 bp, 290 bp and 308 bp respectively (Fig 3), in agreement with the literature (Sumi et al., 2001; Majumder et al., 2008). DNA extracted from bands in agarose gels could be reamplified under the same conditions for cDNA amplification. DNA obtained had concentrations ranging from 10-30 ng μL^{-1}. Reamplification yielded specific bands with no extra bands of any kind.

7.2 Sequencing and Analysis.

Sequences obtained from Functional BioScience laboratories were processed using MEGA 5.1 software (Tamura et al., 2011) and consensus sequences (Appendix F) were attained using both reverse and forward primers sequences. The LYSV sequence had 191 bp and showed 96% identity with a viral sequence from Argentina (AY007693) and 87% to 95% with other LYSV sequences available at GenBank.

The OYDV sequence had 285 bp and showed 94% identity with a viral sequence from Australia that had query coverage of 91% (JN127344), and 80% to 88% with other OYDV sequences available at GenBank with query coverage of 99-100%. The SLV sequence had 308 bp and showed 95% identity with a viral sequence from Shandong province, China (AJ409316) and 78% to 94% with other SLV sequences available at GenBank. The reported similarities are slightly different from the similarities inferred from the trees.

The NSM used for Bayesian phylogenetic analyses was: for LYSV, K80 (Kimura, 1980) with gamma-shaped distribution of rates across sites, for the OYDV coding region, K80 (Kimura, 1980) with gamma-shaped distribution of rates across sites, and for the OYDV non-coding region, SYM (Zharkikh, 1994) with gamma-shaped distribution of rates across sites was used. Both trees were midpoint rooted as sequences most probably evolve at the same rate. The NSM used for SLV analysis was GTR (Tavaré, 1986) with gamma-shaped distribution of rates across sites plus invariant sites. The SLV tree was rooted using the nerine latent virus (NLV) outgroup, which gave the same result as midpoint rooting the tree. Every parameter had a PSRF around 1.00 and it was estimated with an ESS>200. Trace files showed good mixing and convergence of all parameters for each tree. Alignment matrices contained 192, 302 and 310 nucleotides for LYSV, OYDV and SLV respectively.

The LYSV tree clearly grouped virus isolates in two big clades corresponding to China and Japan. Other sequences showed a lower geographical-wise grouping in the phylogenetic analysis. The Ecuadorian LYSV sequence showed a high phylogenetic correlation with leek isolates (Fig 5) and grouped with the Japanese clade and not the Chinese clade. The OYDV sequence reported in this study grouped with onion isolates of the virus (Fig 6). The SLV sequence was more similar to garlic isolates than to isolates from other *Allium* species (Fig 7). The Ecuadorian isolates of LYSV and SLV were grouped together with Indonesian isolates of the same viruses, and the three viruses detected in this study showed high similarity with Argentinian isolates. The biggest clades in the tree are the most probable whereas the smallest clades lack enough phylogenetic signal to be considered real.

7.3 Standardized Micropropagation Protocol

Stem disc identification inside the garlic clove was essential (Fig 8A). Stem discs yielded shoots which formed bulblets after 6-8 weeks of shoot extraction (Fig 8). Shoots that were not extracted directly formed bulblets after 5-7 weeks of culture. The shoot yields were highly dependent on the cutting technique of discs and shoots. MS media was also used, but it yielded no shoots and bulblets as it apparently induced callus formation rather than shoot formation (Fig 9A). Fungicide use was also dropped as the culturing technique was further developed because it was not essential and could have been an additional stress factor for explants.

7.3.1 Stem Disc Culture.

Initially the stem disc was not recognized and so the basal area of the foliar primordium was cut into 2-3 sections. This facilitated the identification of the stem disc and also demonstrated the importance of the basal part of the foliage leave for the appearance of multiple shoots. The use of the steresocopic microscope was essential for the identification of the stem disc. Shoots started appearing 2 to 3 weeks after culturing the stem disc (Fig 9B-C). Cutting the main shoot in the stem disc was vital for the disruption of apical dominance (Fig 8B) which enabled the stem disc to produce multiple shoots (Fig 10). Once the stem disc was properly identified, every correctly cultured stem disc yielded at least 1 shoot. Cutting stem discs in half favored the appearance of more shoots per disc (13 on average) as uncut discs often yielded only 1 shoot. When the stem disc was cut in four parts the total shoot and bulblet yield was effectively nullified.

7.3.2 Shoot Extraction.

An average of 13 shoots (2-40 range) were obtained from each of the 37 cultured stem discs used for this experiment. From the resulting 490 shoots, 41% formed bulblets (199 bulblets) after extraction (Table 2). Bulblets were left to dry for at least 2 weeks and then stored at 4ºC for at least 1 month before being planted in soil. More shoots appeared on the surface of discs which conserved the grooves of the basal foliage leaves. Shoot length at extraction ranged from 0,5 cm to 15 cm. Shoots developed a strong root system and grew 2-3 leaves after 6 to 8 weeks of culture (Fig 11).

7.3.3 Direct Bulb Formation.

95% of shoots left on stem discs formed bulblets after 5 to 7 weeks of shoot appearance (Fig 12); for a total of 124 bulblets (Table 3). 87 bulblets were dried for approximately 2 weeks and then stored at 4ºC for at least 1 month. The other 37 bulblets sprouted without a vernalization period and consequently were planted in pots directly. Green-colored roots grew very close to the base of the bulb, but they developed from the stem disc; true roots pierced through the stem disc and were always white.

7.3.4 Transference to soil.

87 plantlets from the shoot extraction procedure and 43 bulblets from the direct bulb formation procedure were transferred to soil in plant pots. After 4-6 months, bulblets were recovered from 65 plants of the shoot extraction procedure and from 37 plants from the direct bulb formation procedure. Bulblets sprouted into viable plants if the bulblet diameter was approximately 5 mm or more. Plantlets grew more vigorously with the addition of fertilizers (Fig 11). Without them, leaves elongated and fell to the ground. The 102 bulblets bulblets recovered from the first planting in soil were stored at 4ºC for at least 1 month and 50 of them were eventually planted in pots. All 50 bulblets were able to sprout and form new plants (Fig 8 E-F) which in turn enlarged the bulblet size. Cutting leaves for RNA extraction during the transference to soil greatly reduced the plantlet's ability to survive.

7.4 Virus Elimination Diagnosis

59 plants were diagnosed out of the 199 produced by the shoot extraction procedure. 3 plants were LYSV-free, shoot length at extraction was 1 cm on average (0.5-2 range). 2 plants were OYDV-free, shoot length at extraction was 0.8 cm on average (0.5-1 range). 21 plants were SLV-free, shoot length at extraction was 4.4 cm on average (0.5-8 range). All OYDV-free plants were also SLV-free. 1 plant was freed from all 3 viruses diagnosed, shoot length at extraction was 0.5 cm (Table 4).

24 plants were diagnosed out of the 124 produced by the direct bulb formation procedure. 2 plants were LYSV-free, 4 plants were SLV-free and no plant was found OYDV-free from the direct bulb formation process. Similarly, no plant was cleaned from more than 1 virus by the direct bulb formation process (Table 4).

8. DISCUSSION

Viral infection of commercial cultivars appears to be the rule worldwide; this constant has been pointed out in: Germany, Argentina, Belgium, Canada, Czech Republic, Korea, Chile, China, Spain, United States, Philippines, France, England, Japan, Marrueco, New Zealand, Netherlands, Russia, Taiwan, Uruguay, Brazil, Venezuela and Yugoslavia (Conci, 1997). Ecuador is no exception, and we report for the first time the presence of the 3 most important viruses in garlic multiple viral disease: LYSV, OYDV and SLV, in garlic grown in Tumbaco, Pichincha province. When plants were first evaluated in USFQ's fields we found the majority of them with visible symptoms of the viral disease (Fig 13), although there were also some symptomless plants.

Traditionally, the enzyme-linked immunosorbent assay (ELISA) has been the diagnosis tool of choice for large scale routine testing of viruses infecting alliums (Dovas, et al., 2001; Conci, et al., 2003). However, this method has been shown to be problematic due to: the plethora of serotypes that arise from each virus species (Barg, 1996; Tsuneyoshi et al., 1998a; Tsuneyoshi et al., 1998b) as a result of the variable epitope-containing N terminus (Shukla et al., 1988), the low sensitivity of immunosorbent assays when compared to PCR-based methods, and the cross-reactivity of antisera (Dovas, et al., 2001; Shiboleth et al., 2001; Salomon, 2002). Therefore, in this study we took the first steps towards the development of an efficient, sensitive and species-specific molecular diagnosis tool based on PCR.

RNA extraction resulted in hard-to-handle, oily RNA solutions when more than 15 g of garlic tissue was used in the homogeneization step; instead of a pellet, an oily substance formed in the RNA precipitation step (see Appendix C). This was more prone to happen when bulb tissue was used in contrast to leaf tissue, probably because of the former's higher mucilage content (Bos, 1981; Malviya, 2011). This oily substance did form a pellet with the addition of EtOH, but these pellets were harder to dissolve in DEPC-treated water, consistent with polysaccharide (mucilage) contamination (Sambrook & Russell, 2001).

Organic compound contamination was reflected in the sample's low 260/230 index, but phenolate contamination failed to be shown on the 260/280 index. The 1.8 average reported here has been shown to correspond to only 40% RNA, with the remainder being accounted for by protein. Phenol is also known to significantly contribute to overestimation of nucleic acid concentration (Sambrook & Russell, 2001; Bustin & Nolan, 2004, in Fleige & Pfaffl, 2006).

Electrophoretic assesment of RNA samples was less sensitive than nanodrop, and there were samples that amplified OYDV and LYSV even when their respective RNAs were not visible on agarose gels, their concentrations were lower than 30 ng/µL and had a 260/280 index lower than 1.6 and a 260/230 index lower than 0.06. This suggests robustness of the RT-PCR reaction to organic contamination. The positive correlation between amount of tissue used for extraction and concentration of RNA, protein and organic purity obtained is clearly supported by Kendall's tau-b and Spearman's rho (Table 1), although the magnitude of the correlation is low and should be considered significant only in the 10-200 mg range tested.

Pearson's r should be interpreted with caution as the variables mentioned most probably do not have a normal bivariate distribution, thus not fullfilling the assumptions of Pearson's product moment correlation coefficient.

The faint bands obtained for SLV amplification were most probably because of the high annealing temperature used for the thermocycling reaction. We found an optimal annealing temperature for single PCR at 53ºC even when the reported temperature for this primer was 46ºC (Majumder et al., 2008), but for the triplex-PCR we used an annealing temperature of 57ºC. Such an increase in temperature was cushioned by the increment in dNTP's, $MgCl_2$ and buffer concentration (Appendix A).

The highly unresolved trees seen for the reconstructed phylogeny of OYDV and SLV (Fig 6 and 7) were most probably due to the lack of phylogenetic signal to discriminate each sequence from each other, i.e. the nucleotide length used for these two analyses was insufficient to discriminate each virus isolate. In consequence the polytomies observed in the trees generated by Bayesian analysis are most definitely soft-polytomies. Furthermore, each partition of the OYDV sequence had conflicting phylogenetic signals, as shown by generating trees for the coding sequence and the non-coding sequences independently; these conflicting signals can also lead to unresolved phylogenies.

The Ecuadorian LYSV sequence forming a clade with mostly leek isolates and not garlic isolates illustrates the not so restricted range of this potyvirus.The Ecuadorian OYDV sequence showed more similarity to onion isolates than to garlic isolates, this probably has relation to the unsuccesful amplification of the primers O1 and O2, designed to amplify garlic strains of OYDV. These findings are in disagreeance with the recent report of Celli et al. (2013) of garlic and onion-specific strains of OYDV with no cross infectivity. They report nucleic acid sequence divergences enough to separate these strains as different species, according to the ICTV criteria. This disagreement illustrates the genetic diversity present in potyviruses which allows the widely reported host range plasticity and challenges the species definition by ICTV.

The research conducted by Nagasawa & Finer, and Xue et al. (1988; 1991) hinted at the usefulness of the basal part of the garlic clove as a potent explant This fact was further exploited by Ayabe and Sumi (1998). They developed an efficient tissue culture technique suitable for micropropagation and the reported stem-disc dome culture (SD-Dome Culture) technique, capable of producing virus-free garlic bulblets (Ayabe & Sumi, 2001). This technique was adapted for the present study. The most difficult part of adapting the technique was the identification of the stem disc tissue. The null shoot yield of MS media in comparison to LS media is concordant with the callous-inducing properties of MS reported in the literature (Bhojwani et al., 1982; Peña-Iglesias & Ayuso, 1983; Bertaccini et al., 1986; Walkey et al., 1987; Walkey & Antill, 1989).

The 0.85 bulblet formation rate for shoot extraction assays 6 and 7 (Table 3) was mainly due to contamination that originated from the method used to label each individual clove. Labeling required individual sterilization of each clove in separate beakers during culturing in laminar flow; as a high number of cloves were cultured in each assay, laminar flow was obstructed by the numerous beakers in the cabinet. Around half of the cultured stem discs had bacterial or fungal contamination. On the other hand, assays 4 and 5 were carried out with the technique perfected, and had a bulblet formation rate of 1.3. Taking the contamination into account, we can report that the direct bulb formation procedure yields more than one bulblet per disc when the appropiate cutting technique is employed and asceptic conditions are mantained.

The importance of keeping the basal foliage leaves in the stem disc reported by Ayabe and Sumi (2001) were confirmed when stem discs were cut into transversal sections before culturing. Cutting the nascent shoot stimulated multiple shoot appearance and multiple bulb formation from a single stem disc. Ayabe and Sumi (2001) report dividing the stem disc in four parts, but this was impractical for the tissue culture procedure of this study as the variety of garlic we used was smaller and stem discs cut in more than 2 parts did not yield shoots. They also reported shoot yield of 15-25 shoots per stem disc with a bulblet formation rate of more than 90%. We could achieve an average of 13 shoots per stem disc with a bulblet formation rate of 41%, less than half the value of Ayabe and Sumi's report. The comparison is made for stem discs derived from bulblets stored for 8 weeks at 4ºC. It is important to highlight that the efficiency of virus-free plant propagations has been shown to be cultivar-dependent (Koch et al., 1995; Verbeek et al., 1995; Ucman et al., 1998; Shiboleth et al., 2001).

Before discussing virus elimination, we must clarify that by virus-free plants we refer specifically to plants that did not produce amplicons for OYDV, SLV and LYSV by the RT-PCR procedure, performed by duplicate from the RNA extraction step. Samples for RNA extraction were taken from young leaves after the tissue culturing procedure for the diagnosis of presence or absence of viral RNA.

Bulb meristematic tissue (stem disc) has been found to contain virus-free cells from which virus-free plant can be derived (Ayabe & Sumi, 2001). The main reasons attributed to the escape of the meristems by virus invasion are: lack of vascular tissue, which limits viral infection to cell-to-cell movement only; high metabolic activity and the consequent continuous mitosis, which hinders RNA synthesis and hijacking of cellular machinery; high auxin concentration in meristematic cells, plenty of evidence has been found that shows viral-replication inhibition when relatively high levels of auxin are present (Conci, 1997; Razdan, 2003).

Research carried out in tobacco mosaic virus (TMV) found that virions advance at a 5-15 µm/h rate in cell-to-cell movement, which involves intra and intercellular movement (Uppal, 1934 in Conci, 1997). Plant cells have 100 µm on average and divide approximately once per day. This fact combined with the reasons previously stated clarify that cells containing high concentration of auxin, dividing actively and isolated from the vascular system have the highest probability of being virus free.

The molecular mechanism of cell-to-cell movement must also be considered for each specific virus: Carlaviruses (SLV) have potex-like TGB proteins (Fig 1) while Potyviruses (OYDV and LYSV) use the cylindrical inclusion (CI) protein and the trans-frame protein P3N-PIPO (Fig 1) for transport to and across the PD. Perhaps more importantly, potex-like TGB proteins are thought to hijack the cell's actomyosin motility system to deliver ribonucleoprotein complexes (TGB1-CP-genomic RNA) to the PD (Verchot-Lubiczet al., 2010). On the other hand, potyviruses have been found to use the secretory pathway to deliver virions to and through PD and not the actomyosin pathway (Revers et al., 1999; Wei et al., 2010). Both virus families' proteins increase the size exclusion limit of PD, i.e. they make the PD pore larger to allow the passage of bigger molecules.

The shoot extraction experiment shows the elimination of potyviruses only for shoots extracted at a maximum height of 2 cm, while the shallot latent carlavirus is eliminated up to shoots extracted at a height of 8 cm (Table 4). Ayabe and Sumi (2001) have shown that 1cm shoots have developing vascular tissue, thus allowing viral particles to enter by long distance movement mechanism, which also involves the proteins for cell-to-cell movement. However, we found a certain degree of isolation between shoots developing into bulblets and the stem discs that supported them. Nascent roots from developing bulblets pierced through the stem disc while the stem disc developed roots of its own. Moreover, directly formed bulblets detached from the stem disc with ease, suggesting no vasculature between the bulblet and the stem disc. Some of these bulblets were found virus free, even though they were never removed from the stem disc.

Accordingly, we believe that the development of a shoot recruits more meristematic cells than the appearance of dome structures (shoots of less than 1cm) and thus increases the probability of recruiting infected meristematic cells. This would mean that vascular tissue development is not directly involved in viral infection of the dome structures. This is supported by the fact that the direct bulb formation process was capable of producing virus-free plantlets (Table 4). It did so with the same virus family elimination bias as the shoot extraction process. Moreover, some studies have found that a single garlic head may contain virus-free and virus-infected cloves (Ramírez-Malagon et al., 2006; Conci et al., 2010), which also suggest that the cluster of meristematic cells may contain infected and clean cells from which shoots and bulblets develop.

Under this model, the higher virus elimination rate for the shoot extraction procedure, in comparison to the direct bulb formation procedure, would stem from the idea that a developing shoot actively recruits meristematic cells until it reaches certain maturity and isolates itself from the stem disc. This would increase the probability of recruiting infected cells as the shoot increases in length, explaining the result of Ayabe and Sumi (2001) that a shoot of 1 cm or more in length would be expected to be infected by LYSV. We monitored 3 viruses instead of 1 and found that the size limit for generating a virus-free plant is higher for the shallot latent carlavirus (8 cm). Ayabe and Sumi (2001) also suggest that plasmodesmata may not be fully matured during the development of dome structures, thus hindering the ability of viruses to infect these cells.

The differential elimination bias, related to viral family, probably stems from the different mechanisms of their MPs. Carlaviruses use microfilaments and the associated myosin motor molecules (Verchot-Lubiczet al., 2010) while potyviruses use microtubules exclusively and the kinesin motors associated with these structures (Revers et al., 1999; Wei et al., 2010). Further experiments should assess viral MP function in primary vs secondary (matured) plasmodesmata. The viral elimination bias we report could be further experimented in garlic if the garlic common latent carlavirus is also brought into the analysis. This would bring more evidence to further test the hypothesis presented as a possible explanation of the viral-elimination bias.

9. CONCLUSIONS

- Stem disc culture followed by shoot extraction is an effective method for eliminating viruses in garlic and propagating virus-free bulblet seeds.
- Culturing shoots before they reach 0.5 cm of height is critical for the production of virus-free bulblets.
- Hormone-free LS media is a better medium for shoot production from garlic stem discs than MS.
- The basal parts of foliage leaves are essential for the appearance of shoots in the stem disc.
- The main shoot in the stem disc must be cut to disrupt apical dominance and promote multiple shoot appearance.
- Triplex RT-PCR amplified the targeted viral sequences and thus it is a useful diagnosis tool.
- LYSV and OYDV were detected down to 30ng of total RNA, while SLV detection was less sensitive; being detected down to 60ng of total RNA.
- There was a bias towards the preferential elimination of SLV independent of the method used to produce a bulblet.
- The direct bulb formation process was capable of producing partially virus-free plants, although with less efficiency than the shoot extraction procedure.

10. RECOMMENDATIONS

- Diagnosis for Garlic Common Latent carlavirus (GarCLV) could be implemented to test the hypothesis of viral differential elimination being due to the different nature of movement proteins of each virus family. Likewise, allexiviruses could also be diagnosed to further test the hypothesis.
- Electrotherapy could be given to bulbs prior to cold storage to improve the virus removal rate without significantly increasing labor.
- An intermediary phase between shoot extraction and transference to soil, where the plantlet would be gradually acclimatized to less humidity, until ambient humidity is reached, could improve the percentage of bulblet formation. The photoperiod should also gradually resemble the greenhouse photoperiod.
- Individual labeling of cloves during culturing should be avoided to avoid contamination. If necessary, small amounts of labeled cloves should be cultured in batches to circumvent blocking the laminar flow with a high amount of beakers.
- It is very important, for a correct diagnosis, to use less than 15 g of a full grown garlic leave in the homogenization step to avoid an oily RNA extract and produce a normal pellet.
- Using approximately 100 mg of tissue for the extraction seems to work best in terms of RNA yield and purity obtained.
- More RNA could be used during reverse transcription to test if the sensibility for SLV detection is increased, along with a minor decrease in the annealing temperature in the triplex-PCR.

- A one-step RT-PCR methodology could be implemented alongside multiplexing to further reduce the time needed to execute the test.
- Adding a primer that amplifies a garlic constitutive mRNA would serve as a control for the diagnosis procedure.
- Real time qPCR would help determine viral load by also amplifying a normalizing garlic mRNA gene. This would reduce the time needed to visualize results as electrophoresis in agarose gels would no longer be needed.

11. BIBLIOGRAPHY

Abo El-Nil, M. (1977). Organogenesis and embryogenesis in callus cultures of garlic (*Allium sativum* L.). *Plant Science Letters, 9*, 259-264.

Altschul, S., Madden, T., Schäffer, A., Zhang, J., Zhang, Z., Miller, W., et al. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research, 25*, 3389-3402.

Anderson, B. (2013). *Gourmet Garlic Gardens*. Retrieved from The Garlic Family Tree and Where Garlic Came from: http://www.gourmetgarlicgardens.com/overview.htm

Ankri, S., & Mirelman, D. (1999). Antimicrobial properties of allicin from garlic. *Microbes and Infection, 2*(2), 125–9.

Atreya, C., Raccah, B., & Pirone, T. (1992). A point mutation in the coat protein abolishes aphid transmissibility of a potyvirus. *Virology, 178*, 161-165.

Atreya, P., Atreya, C., & Pirone, T. (1991). Amino acid substitutions in the coat protein result in loss of insect transmissibility of a plant virus. *Proceedings of the National Academy of Sciences of the United States of America, 88*, 7887–7891.

Ayabe, M., & Sumi, S. (1998). Establishment of a novel tissue culture method, stem-disc culture, and its practical application to micropropagation of garlic (*Allium sativum* L.). *Plant Cell Reports, 17*, 773-779.

Ayabe, M., & Sumi, S. (2001). A novel and efficient tissue culture method - "stem-disc dome culture" - for producing virus-free garlic (*Allium sativum* L.). *Plant Cell Reports, 20*(6), 503-507.

Banerjee, S., Mukherjee, P., & Maulik, S. (2001). Garlic as an Antioxidant: The Good, The Bad and The Ugly. *Phytotherapy Research, 17*(2), 97–106.

Barg, E. (1996). Serologische und molekulargenetische Untersuchungen zur Variabilität Allium-Arten infizierender, filamentöser Viren. *Papierflieger*.

Bautista, D., Movahed, P., Hinman, A., Axelsson, H., Sterner, O., Hogestatt, E., et al. (2005). Pungent products from garlic activate the sensory ion channel TRPA1. *Proclamations of the National Academy of Science, 102*(34), 12248–52.

BCE. (2013, March). *Banco Central del Ecuador*. Retrieved from Comercio Exterior: http://www.portal.bce.fin.ec/vto_bueno/seguridad/ComercioExteriorEst.jsp

Bertaccini, A., Marani, F., & Borgia, F. (1986). Shoot-tip culture of different garlic lines for virus elimination. *Revista Ortoflorofrutticoltura Italiana, 70,* 97-105.

Bhagyalakshmi, N., Thimmaraju, R., Venkatachalam, L., Chidambara, K., & Sreedhar, R. (2005). Nutraceutical applications of Garlic and the Intervention of Biotechnology. *Critical Reviews in Food Science and Nutrition, 45*, 607-621.

Bhojwani, S., Cohen, D., & Fry, P. (1982). Production of virus-free garlic and field performance of micropropagated plants. *Scientia Horticulturae*, 39-43.

Biomatters. (2013). Geneious R 7 (Beta). http://www.geneious.com/.

Blanc, S. (2008). Vector transmission of plant viruses. In B. Mahy, & M. Regenmortel, *Desk Encyclopedia of Plant and Fungal Virology* (pp. 10-18). Elsevier, Academic Press.

Bos, L. (1976). *Onion yellow dwarf virus.* Retrieved from Descriptions of Plant Viruses: http://www.dpvweb.net/dpv/showdpv.php?dpvno=158

Bos, L. (1981). *Leek yellow stripe virus.* Retrieved from Descriptions of Plant Viruses: http://www.dpvweb.net/dpv/showdpv.php?dpvno=240

Bos, L. (1982). *Shallot latent virus.* Retrieved from Descriptions of Plant Viruses: http://www.dpvweb.net/dpv/showdpv.php?dpvno=250

Bos, L., Huijberts, N., Huttinga, H., & Maat, D. (1978). Leek yellow stripe virus and its relationships to Onion yellow dwarf virus - characterization, ecology, and possible control. *Netherlands Journal of Plant Pathlogy, 84*(5), 185-204.

Bos, L., Huttinga, H., & Maat, D. (1978). Shallot latent virus, a new carlavirus. *Netherlands Journal of Plant Pathology, 84*(6), 227-237.

Brunt, A., Crabtree, K., Dallwitz, M., Gibbs, A., Watson, L., & Zurcher, E. (1996). *Plant Viruses Online: Descriptions and Lists from the VIDE Database.* Retrieved from Shallot Latent Virus: http://biology.anu.edu.au/Groups/MES/vide

Caciagli, P. (2008). Vegetable Viruses. In B. Mahy, & M. Regenmortel, *Desk Encyclopedia of Plant and Fungal Virology* (p. 484). Elsevier, Academic Press.

Cafrune, E., Perotto, M., & Conci, V. (2006). Effect of Two Allexivirus Isolates on Garlic Yield. *Plant Disease, 90*, 898-904.

Cann, A. (2009). *Plant Viruses*. Retrieved from MicrobiologyBytes: http://www.microbiologybytes.com/virology/Plant.html

Celli, M., Torrico, A., Kiehr, M., & Conci, V. (2013). Striking differences in the biological and molecular properties of onion and garlic isolates of onion yellow dwarf virus. *Archives of Virology, 158*, 1377-1382.

Chen, J., Adams, M., Zheng, H., & Chen, J. (2003). Sequence analysis demonstrates that Onion yellow dwarf virus isolates from China contain a P3 region much larger than other potyviruses. *Archives of Virology, 148*, 1165–1173.

Chen, J., Chen, J., & Adams, M. (2001). Molecular characterisation of a complex mixture of viruses in garlic with mosaic symptoms in China. *Archives of Virology, 146*, 1841-1853.

Chung, B., Miller, W., Atkins, J., & Firth, A. (2008). An overlapping essential gene in the Potyviridae. *Proceedings of the National Academy of Sciences, 105*(15), 5897–5902.

Citovsky, V., & Zambryski, P. (2005). How do plant virus nucleic acids move through intercellular connections? *BioEssays, 13*(8), 373-379.

Conci, V. (1997). Virus y Fitoplasmas de Ajo. *50 Temas sobre producción de Ajo* (pp. 267-284). Mendoza, Argentina: Instituto de Fitopatología y Fisiología Vegetal.

Conci, V., Canavelli, A., & Balzarini, M. (2010). The distribution of garlic viruses in leaves and bulbs during the first year of infection. *Journal of Phytopathology, 158*, 186–93.

Conci, V., Canavelli, A., Lunello, P., Di Renzo, L., Nome, S., Zumelzu, G., et al. (2003). Yield Losses Associated with Virus-Infected garlic Plants during Five Successive Years. *Plant Disease, 87*(12), 1411-1415.

Davis, R. (2008). Garlic Mosaic. In H. F. Schwartz, *Diseases caused by Viruses and Phytoplasmas* (pp. 78-83). St. Paul: American Phytopathological Society.

Dovas, C., Hatziloukas, E., Salomon, R., Barg, E., Shiboleth, Y., & Katis, N. (2001a). Comparison of Methods for Virus Detection in *Allium* spp. *Journal of Phytopathology, 149*, 731-737.

Dovas, C., Hatziloukas, E., Salomon, R., Barg, E., Shiboleth, Y., & Katis, N. (2001b). Incidence of viruses infecting *Allium* spp. in Greece. *European Journal of Plant Pathology, 107*, 677-684.

Elkayam, A., Mirelman, D., Peleg, E., Wilchek, M., Miron, T., Rabinkov, A., et al. (2003). The effects of allicin on weight in fructose-induced hyperinsulinemic, hyperlipidemic, hypertensive rats. *American Journal of Hypertension, 16*(12), 1053-1056.

Elnagar, S., El-Sheik, M., & Abdel Wahab, A. (2009). Effect Of Natural Infection With Onion Yellow Dwarf Virus (Oydv) On Yield Of Onion And Garlic Crops In Egypt. *4 th Conference on Recent Technologies in Agriculture,* (pp. 34-39). Giza.

Ensminger, A. (1994). *Foods & Nutrition Encyclopedia* (Vol. 1). CRC Press.

Etoh, T., & Simon, P. (2002). *Allium Crop Science Recent Advances, Diversity Fertility and Seed Production of Garlic.* (H. &. Rabinowitch, Ed.) CAB International.

Fleige, S., & Pfaffl, M. (2006). RNA integrity and the effect on the real-time qRT-PCR performance. *Molecular Aspects of Medicine, 27,* 126-139.

Fuchs, M. (2008). Plant Resistance to Viruses: Engineered resistance. In B. Mahy, & M. Regenmortel, *Desk Encyclopedia of Plant and Fungal Virology* (pp. 44-52). Elsevier, Academic Press.

GE Healthcare. (2008). First-Strand cDNA Synthesis Kit. Little Chalfont, Buckinghamshire, UK.

Ghoshroy, S., Lartey, R., Sheng, J., & Citovsky, V. (1997). Transport of Proteins and Nucleic Acids through Plasmodesmata. *Annual Revisions in Plant Physiology and Plant Molecular Biology, 48,* 27-50.

Harries, P., & Nelson, R. (2008). Movement of Viruses in plants. In B. Mahy, & M. Regenmortel, *Desk Encyclopedia of Plant and Fungal Virology* (pp. 3-10). Elsevier, Academic Press.

Havránek, P., & Novák, F. (1973). The bud formation in the callus cultures of *Allium sativum* L. *Z Pflanzenphysiol*, 308-318.

Hull, R. (2001). Classifying reverse transcribing elements: a proposal and a challenge to the ICTV. *Archives of Virology, 146*(11), 2255–2261.

Instituto Nacional Autónomo de Investigaciones Agropecuarias (INIAP). (2008). Ajo. In A. Villavicencio, & W. Vásquez, *Guía Técnica de Cultivos.* Quito: Ministerio de Agricultura, Ganadería, Acuacultura y Pesca.

James, D., Varga, A., Pallas, V., & Candresse, T. (2006). Strategies for simultaneous detection of multiple plant viruses. *Canadian Journal of Plant Pathology, 28*(1), 16-29.

Kamenetsky, R., & Rabinowitch, H. (2002). *Allium Crop Science Recent Advances, Florogenesis.* (H. Rabinowitch, & L. Currah, Eds.) CAB International.

Kehr, A., & Schaeffer, G. (1976). Tissue culture and differentiation of. *Horticultural Science, 11*, 422-423.

Keusgen, M. (2002). Health and Alliums. In H. Rabinowitch, & L. Currah, *Allium Crop Science: Recent Advances* (pp. 365-371). CABI Publishing.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution, 16*(2), 111-120.

Koch, M., Ta'anami, Z., & Salomon, R. (1995). Improved regeneration of shoots from garlic callus. *Horticultural Science, 30*, 378.

Linsmaier, E., & Skoog, F. (1965). Organic Growth Factor Requirements of Tobacco Tissue Cultures. *Physiologia Plantarum, 18*, 100-127.

Loebenstein, G. (2008). Plant Virus Diseases: Economic Aspects. In B. Mahy, & M. Regenmortel, *Desk Encyclopedia of Plant and Fungal Virology* (pp. 426-30). Elsevier, Academic Press.

Lot, H., Chovelon, V., Souche, S., & Delecolle, B. (1998). Effects of Onion Yellow Dwarf and Leek Yellow Stripe Viruses on Symptomatology and Yield Loss of Three French Garlic Cultivars. *Plant Disease, 82*, 1381-1385.

Lot, H., Delecolle, B., Boccardo, G., Marzachi, C., & Milne, R. (1994). Partial characterization of reovirus-like particles associated with garlic dwarf disease. *Plant Pathology, 43*(3), 537-546.

Lunello, P., Ducasse, D., Helguera, M., Nome, S., & Conci, V. (2002). An Argentinean isolate of Leek Yellow Stripe Virus from Leek can be transmitted to Garlic. *Journal of Plant Pathology, 84*(1), 11-17.

Lunello, P., Rienzo, J., & Conci, V. (2007). Yield Loss in Garlic Caused by Leek yellow stripe virus Argentinean Isolate. *Plan Disease, 91*, 153-158.

Ma, Y., Whang, H., Zhang, C., & Kang, Y. (1994). High rate of virus-free plantlet regeneration via garlic scape-tip culture. *Plant Cell Reports, 14*, 65-68.

Macpherson, L., Geierstanger, B., Viswanath, V., Bandell, M., Eid, S., & Hwang, S. A. (2005). The pungency of garlic: Activation of TRPA1 and TRPV1 in response to allicin. *Current Biology, 15*(10), 929-934.

Mahmoud, S., Maaty, S., Borollosy, A., & Abdel-Ghaffar, M. (2007). Identification of Onion Yellow Dwarf Potyvirus as one of the Major Viruses Infecting Garlic in Egypt. *American-Eurasian Journal of Agricultural and Enviromental Sciences, 2*(6), 746-755.

Majumder, S., Baranwal, V., & Joshi, S. (2008). Simultaneous Detection Of Onion Yellow Dwarf Virus And Shallot Latent Virus In Infected Leaves And Cloves Of Garlic By Duplex RT-PCR. *Journal of Plant Pathology, 90*(2), 371-374.

Malviya, R., Srivastava, P., & Kulkarni, G. (2011). Applications of Mucilages in Drug Delivery. *Advances in Biological Research, 5*(1), 1-7.

Matsuura, H. (2008). Phytochemistry of Garlic horticultural processing procedures. In P. Lachance, *Nutraceuticals: Designer Foods III: Garlic, Soy and Licorice* (pp. 55-60). Trumbull, Conneticut: Food and Nutrition Press, Inc.

Meenakshi, A., Baranwal, V., Ahlawat, Y., & Singh, L. (2006). RT-PCR detection and molecular characterization of Onion Yellow Dwarf Virus associated with garlic and onion. *Current Science, 91*(9), 1230-34.

Milne, I., Lindner, D., Bayer, M., Husmeier, D., McGuire, G., Marshall, D., et al. (2008). TOPALi v2: a rich graphical interface for evolutionary analyses of multiple alignments on HPC clusters and multi-core desktops. *Bioinformatics, 25*(1), 126-127.

Ministerio de Agricultura y Ganaderia (MAG). (1986). Ajo *Allium sativum* L. In M. d. (MAG), *Inventario de Plagas, Enfermedades y Malezas del Ecuador* (pp. 11-13). Quito.

Myers, J., & Simon, P. (1998). Continuous callus production and regeneration of garlic (*Allium sativum* L.) using root segments from shoot tip-derived plantlets. *Plant Cell Reports, 17*, 726-730.

Nagakubo, T., Nagasawa, A., & Ohkawa, H. (1993). Micropagation of garlic through in vitro bulblet formation. *Plant Cell Tissue Organ Culture, 32*, 175-183.

Nagasawa, A., & Finer, J. (1988). Induction of Morphogenic Callus Cultures from Leaf Tissue of Garlic. *HortScience, 23*(6), 1068-1070.

Peña-Iglesias, A., & Ayuso, P. (1983). Characterization of Spanish Garlic viruses and their elimination by in vitro shoot apex culture. *Acta Horticulturae, 127*, 183-193.

Posada, D., & Crandall, K. (2001). Selecting the Best-FitModel of Nucleotide Substitution. *Systematic Biology, 50*(4), 580-601.

Rambaut, A., & Drummond, A. (2007). *Tracer v1.5. URL: http://beast.bio.ed.ac.uk/Tracer.*

Ramírez-Malagon, R., Pérez-Moreno, L., Borodanenko, A., Salinas-Gonzáles, S., & Ochoa-Alejo, N. (2006). Differential organ infection studies, potyvirus elimination, and field performance of virus-free garlic plants produced by tissue culture. *Plant Cell Tissue Organ Culture, 86*, 103-110.

Razdan, M. (2003). Production of Disease-Free Plants. In M. Razdan, *Introuction to Plant Tissue Culture* (pp. 219-232). Enfield: Science Publishers Inc.

Regenmortel, V. (2008). The nature of viruses. In B. Mahy, & M. Regenmortel, *Desk Encyclopedia of General Virology* (pp. 19-23). Elsevier, Academic Press.

Revers, F., Le Gall, O., Candresse, T., & Maule, A. (1999). New Advances in Understanding the Molecular Biology of Plant/Potyvirus Interactions. *The American Phytopathological Society, 12*(5), 367-376.

Riechmann, J., Lain, S., & Garcia, J. (1992). Highlights and prospects of potyvirus molecular biology. *The Journal of General Virology, 73*, 1-16.

Roberts, A. (2005). Plasmodesmal structure and development. In K. Oparka, *Plasmodesmata* (pp. 1-21). Blackwell Publishing.

Robledo-Paz, A., Villalobos, V., & Jofre-Garfias, A. (2000). Efficient plant regeneration of garlic (*Allium sativum* L.) by root tip culture. *In vitro Cellular and DEvelopmental Biology, 36*, 416-419.

Ronquist, F., & Huelsenbeck, J. (2003). MRBAYES 3: Bayesian phylogenetic. *Bioinformatics, 19*, 1572-1574.

Salomon, R. (2002). Virus Disease in Garlic and the Propagation of virus-free Plants. In B. Mahy, & M. Regenmortel, *Desk Encyclopedia of Plant and Fungal Virology* (pp. 312-324). Elsevier, Academic Press.

Sambrook, J., & Russell, D. (2001). *Molecular Cloning: A Laboratory Manual.* New York: Cold Spring Harbor laboratory Press.

Santa Cruz, S. (1999). Perspective: phloem transport of viruses and macromolecules - what goes in must come out. *Trends in Microbiology, 7*(6), 237-241.

Shiboleth, Y., Gal-On, A., Koch, M., Rabinowitch, H., & Salomon, R. (2001). Molecular characterisation of Onion yellow dwarf virus (OYDV) infecting garlic (*Allium sativum* L.) in Israel: Thermotherapy inhibits virus elimination by meristem tip culture. *Annals of Applied Biology, 138*, 187-195.

Shukla, D., Strike, P., Tracy, S., Gough, K., & Ward, C. (1988). The N and C Termini of the Coat Proteins of Potyviruses Are Surface-located and the N Terminus Contains the Major Virus-specific Epitopes. *Journal of General Virology, 69*, 1497-1508.

Silagy, C., & Neil, H. (1994). A meta-analysis of the effect of garlic on blood pressure. *Journal of Hypertension, 12*(4), 463-468.

Simonetti, G. (1990). *Simon & Schuster's Guide to Herbs and Spices.* Simon & Schuster, Inc.

Soliman, A., Mahmoud, S., & Dawood, R. (2012). Molecular Characterization of Onion Yellow Dwarf Virus (garlic isolate) with production of virus-free plantlets. *International Journal of Virology, 8*(1), 61-70.

SPSS Inc. (2008). SPSS Statistics for Windows, Version 17.0. Chicago: SPSS Inc. .

Srivastava, K. (1986). Evidence for the mechanism by which garlic inhibits platelet aggregation. *Prostaglandins, Leukotrienes and Medicine, 22*(3), 313–321.

Sullivan, M., & Robinson, A. (2012). *CPHST Pest Datasheet for Leek Yellow Stripe virus.* USDA.

Sumi, S., Tsuneyoshi, T., Susuki, A., & Ayabe, M. (2001). Development and Establishment of Practical Tissue Culture Methods for Production of Virus-Free Garlic Seed Bulbs, a Novel Field Cultivation System and Convenient Methods for Detecting Garlic Infecting Viruses. *Plant Biotechnology, 18*(3), 179-190.

Sutic, D., Ford, R., & Tosic, M. (1999). Onion yellow dwarf virus. In *Handbook of Plant Virus Diseases* (pp. 158-9). CRC Press.

Swiss Institute of Bioinformatics. (2013). *Potyvirus, Carlavirus, Betaflexiviridae.* Retrieved from Viral Zone: www.expasy.org/viralzone

Takaki, F., Sano, T., & Yamashita, K. (2006). The complete nucleotide sequence of attenuated onion yellow dwarf virus: a natural potyvirus deletion mutant lacking the N-terminal 92 amino acids of HC-Pro. *Archives of Virology, 151*, 1439–1445.

Takaki, F., Sano, T., Yamashita, K., Fujita, T., Ueda, K., & Kato, T. (2005). Complete nucleotide sequences of attenuated and severe isolates of Leek yellow stripe virus from garlic in northern Japan: Identification of three distinct virus types in garlic and leek world-wide. *Archives of Virology, 150*, 1135-1149.

Tamura, K., & Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution, 10*(3), 512–526.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary

Distance and Maximum Parsimony Methods. *Molecular Biology and Evolution, 28*(10), 2731-2739.

Tavaré, S. (1986). Some Probabilistic and Statistical Problems in the Analysis of DNA Sequences. *Lectures on Mathematics in the Life Sciences (American Mathematical Society), 17*, 57-86.

Thompson, J., Higgins, D., & Gibson, T. (1994). CLUSTAL W: improving the sensitivity of progressice multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research, 22*(22), 4673-4680.

Tsuneyoshi, T., Matsumi, T., Deng, T., Sako, I., & Sumi, S. (1998a). Differentiation of *Allium* Carlaviruses isolated from different parts of the world based on the viral coat protein sequence. *Archives of Virology, 143*, 1093-1107.

Tsuneyoshi, T., Matsumi, T., Natsuaki, K., & Sumi, S. (1998b). Nucleotide sequence analysis of virus isolates indicates the presence of three potyvirus species in *Allium* plants. *Archives of Virology, 143*, 97-113.

Ucman, R., Zel, J., & Ravnikar, M. (1998). Thermotherapy in virus elimination from garlic: influences on shoot multiplication from meristems and bulb formation in vitro. *Scientia Horticulturae, 73*, 193-202.

UN Food & Agriculture Organization (FAO). (2011). *Major Food And Agricultural Commodities And Producers - Countries By Commodity.*

Van Dijk, P. (1993a). Survey and characterization of potyviruses and their strains of *Allium* species. *Netherlands Journal of Plant Pathology, 99*(2), 1-48.

Van Dijk, P. (1993b). Carlavirus isolates from cultivated *Allium* species represent three viruses. *Netherlands Journal of Plant Pathology, 99*, 233-257.

Van Regenmortel, M., Fauquet, C., Bishop, D., Carstens, E., Estes, M., Lemon, S., et al. (2000). *Virus Taxonomy. Seventh Report of the International Committee on Taxonomy.* London: Academic Press.

Verbeek, M., Van Dijk, P., & Van Well, P. (1995). Efficiency of eradication of four viruses from garlic (*Allium sativum*) by meristem tip-culture. *European Journal of Plant Pathology, 101*, 231-239.

Verchot-Lubicz, J., Torrance, L., Solovyev, A., Morozov, S., Jackson, A., & Gilmer, D. (2010). Varied Movement Strategies Employed by Triple Gene Block-Encoding Viruses. *The American Phytophatological Society, 23*(10), 1231-1247.

Walkey, D., & Antill, D. (1989). Agronomic evaluation of virus free and virus infected garlic (*Allium sativum* L.). *Journal of Hrticultural Science, 64*, 53-60.

Walkey, D., Webb, M., Bolland, C., & Miller, A. (1987). Production of virus free garlic (*Allium sativum* L.) and shallot (A. ascalonicum L.) by meristem-tip culture. *Journal of Horticultural Science, 62*, 211-220.

Washington State University. (2013). *Garlic Scapes.* Retrieved from Vegetable Research and Extension: http://agsyst.wsu.edu/GarlicScapes.html

Wei, T., Pearson, M., & Fletcher, J. (2006). Molecular confirmation of New Zealand garlic yellow streak virus as leek yellow stripe virus. *Australasian Plant Pathology, 35*, 341-346.

Wei, T., Zhang, C., Hong, J., Xiong, R., Kasschau, K., Zhou, Z., et al. (2010). Formation of complexes at Plasmodesmata for Potyvirus Intercellular movement is mediated by the viral protein P3N-PIPO. *Plos Pathogens, 6*(6), 1-12.

Wylie, S., Luo, H., Li, H., & Jones, M. (2012). Multiple polyadenylated RNA viruses detected in pooled cultivated and wild plant samples. *Archives of Virology, 157*(2), 271-284.

Xue, H., Araki, H., Shi, L., & Yakuwa, T. (1991). Somatic embryogenesis and plant regeneration in basal plate and receptacle-derived callus cultures of garlic (*Allium sativum* L.). *Japan Society of Horticultural Science, 60*(3), 627-634.

Zaitlin, M., & Palukaitis, P. (2000). Advances in understanding plant viruses and virus diseases. *Annual Revisions in Phytopathology, 38*, 117-43.

Zharkikh, A. (1994). Estimation of phylogenetic distances between nucleotides. *Journal of Molecular Evolution, 39*, 315-329.

Zohary, D., & Hopf, M. (2000). *Domestication of plants in the Old World* (3rd ed.). Oxford University Press.

12. TABLES

Table 1: Correlation coefficients between the amount of tissue used for the RNA extraction and the amount and quality of RNA obtained in a 10-200 mg range

			260/230	[RNA]	260/280
Kendall's tau-b	Amount of Tissue	Correlation Coefficient	.188**	.116*	.350**
		Sig. (2-tailed)	.000	.027	.000
		N	172	172	172
Spearman's rho	Amount of Tissue	Correlation Coefficient	.291**	.171*	.512**
		Sig. (2-tailed)	.000	.025	.000
		N	172	172	172
Pearson's r	Amount of Tissue	Correlation Coefficient	.209**	.190*	.458**
		Sig. (2-tailed)	.006	.012	.000
		N	172	172	172

N: sample size; Sig.: p-value for the hypothesis test **: correlation is significant at the 0.01 level (2-tailed); *: correlation is significant at the 0.05 level (2-tailed). Tests carried out using SPSS v17 software (SPSS Inc., 2008)

Table 2: Shoot yields from 37 garlic stem discs at 3 different shoot extraction events

Stem Disc	1st Shoot Extraction	2nd Shoot Extraction	3rd Shoot Extraction	Total
A	9	-	-	9
B	8	1	-	9
C	11	11	2	24
D	1	1	10	12
E	8	2	-	10
F	13	10	4	27
G	11	1	-	12
H	4	-	-	4
I	4	1	-	5
J	5	3	-	8
K	2	-	-	2
L	2	-	-	2
M	11	1	1	13
N	8	8	-	16
O	6	7	-	13
P	7	3	1	11
Q	10	-	-	10
R	10	-	-	10
S	16	4	-	20
T	10	-	-	10
U	10	-	-	10
V	10	-	-	10
W	6	-	-	6
X	4	-	-	4
Y	10	5	-	15
Z	8	6	-	14
AA	13	3	-	16
AB	13	12	-	25
AC	13	-	-	13
AD	19	11	11	41
AE	11	10	10	31
AF	10	1	-	11
AG	10	-	-	10
AH	12	10	4	26
AI	9	5	-	14
AJ	9	-	-	9
AK	8	-	-	8
			Total	490
			Mean	13.24

The letters in the "Stem Disc" column served as identification for each stem disc cultured and this nomenclature was carried on into further experiments. Stem discs produced shoots continuously and thus shoots were harvested accordingly.

Table 3: Bulblet yields from 7 stem disc culture assays under the direct bulb formation procedure

Assay #	Discs Extracted	Discs Correctly Cultured	Bulblets	Average bulblet Production per disc	Observations
1	9	0	0	-	Stem disc was not correctly identified
2	11	0	0	-	Stem disc was not correctly identified
3	30	23	12	0.52	Stem disc cut in transversal sections in four different ways
4	30	15	21	1.40	MS vs. LS* media test
5	32	32	40	1.25	None
6	18	8	5	0.63	Heavy contamination due to individual sterilization
7	65	52	46	0.89	Heavy contamination due to individual sterilization
Total	195	130	124	0.95	

Discs correctly cultured: the amount of discs extracted minus the amount of discs later found to be incorrectly cultured due to using the wrong cutting technique or due to contamination. *: see Appendix B

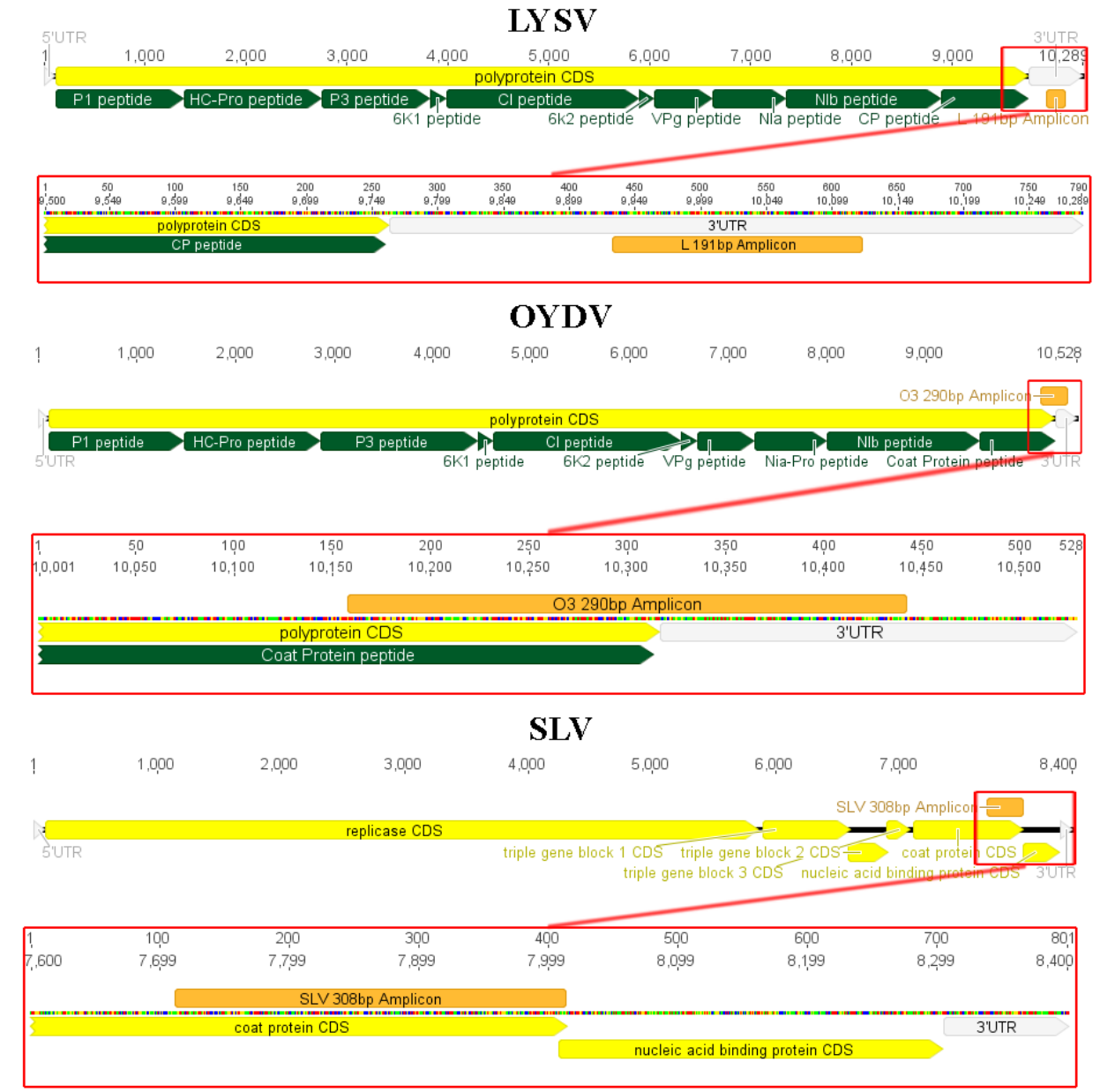
Table 4: Virus elimination diagnosis results for garlic explants cultured by two different methods

		Before Tissue Culture			After Tissue Culture			
Method	**ID**	**SLV**	**OYDV**	**LYSV**	**SLV**	**OYDV**	**LYSV**	**Shoot Size [cm]**
Shoot Extraction	A1	+	+	+	-	+	+	5
	AA1	+	+	+	-	+	-	0.5
	AA13	+	+	+	-	+	+	3
	AH22	+	+	+	-	+	+	5
	Ai2	+	+	+	-	+	+	5
	AK1	+	+	+	-	+	+	4
	AK3	+	+	+	-	+	+	5
	B4	+	+	+	-	+	+	3
	C3	+	+	+	-	+	+	8
	E6	+	+	+	-	+	+	5
	H4	+	+	+	-	+	+	5
	J2	+	+	+	-	+	+	4
	P4	+	+	+	-	+	+	8
	P5	+	+	+	-	+	+	4
	S3	+	+	+	-	+	+	8
	S4	+	+	+	-	+	+	7
	V2	+	+	+	-	+	+	3
	V9	+	+	+	-	+	+	6
	81	+	+	+	-	-	-	0.5
	85	-	+	+	-	+	-	2
	86	+	+	+	-	+	+	2
	99	+	+	+	-	-	+	1
							N=199	**n**=59
Direct Bulb Formation	44	+	+	+	+	+	-	n/a
	45	+	+	+	-	+	+	n/a
	46	-	+	+	-	+	-	n/a
	76	+	+	+	-	+	+	n/a
	70	+	+	+	-	+	+	n/a
	95	+	+	+	-	+	+	n/a
							N=124	**n**=24

ID: identification, letters follow the labeling system for the shoot extraction experiment described in Table 2 with the added number describing a unique individual. Numbers in the ID column describe unique stem discs from assays 1-7 described in Table 3. Samples 81, 85, 86 and 99 were special cases of stem discs that had shoots extracted but were not considered for the shoot extraction experiment due to the technique not being completely standardized. Shoot size: size in cm of shoots at extraction. SLV: shallot latent virus; LYSV: leek yellow stripe virus; OYDV: onion yellow dwarf virus; (+): positive according to multiplex RT-PCR performed by duplicate; (-): negative according to multiplex RT-PCR performed by duplicate; N: total bulblets produced by the in vitro culturing method; n: number of bulblets diagnosed from the total N. SLV negative samples were re-confirmed by single RT-PCR.

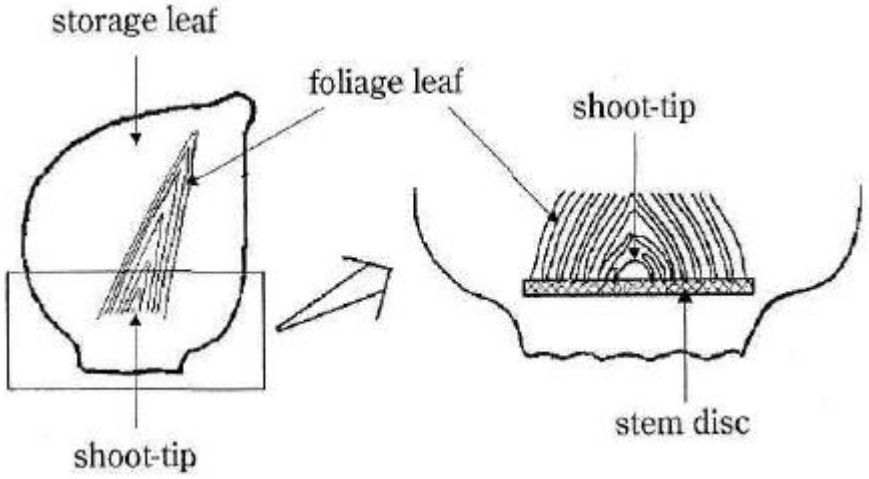
13. FIGURES

Figure 1: Target regions for amplification of 3 viral genomes with primers L, O3 and S



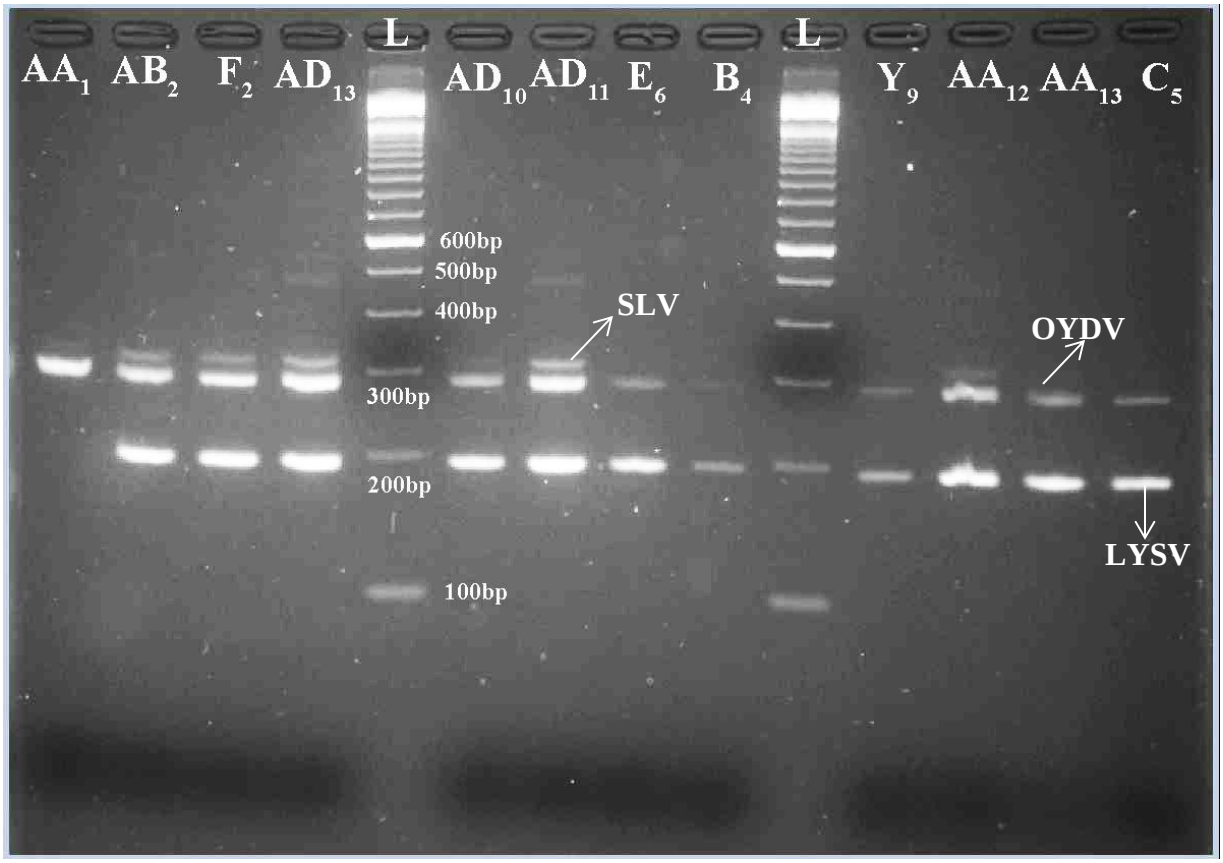
Potyviral genomes (LYSV and OYDV) are of 10kb in size and encode a single polyprotein which is auto-cleaved to yield the other proteins needed for the virus life cycle. **LYSV:** the L primer amplified 191 bp from the 3' UTR of the viral genome. **OYDV:** the primer O3 amplified 290 bp from the coat protein (CP) gene and part of the 3' UTR. **SLV:** the S primer amplified 308 bp from the 3' end of the CP gene. The SLV genome is of 8.4 kb in size and encodes triple gene block proteins which allows cell-to-cell and long-distance movements. For complete information regarding primers see Appendix F. Images generated using Geneious version 7 (Biomatters, 2013). LYSV: leek yellow stripe virus; OYDV: onion yellow dwarf virus; SLV: shallot latent virus.

Figure 2: Schematic depiction of garlic tissue designated the "stem disc"



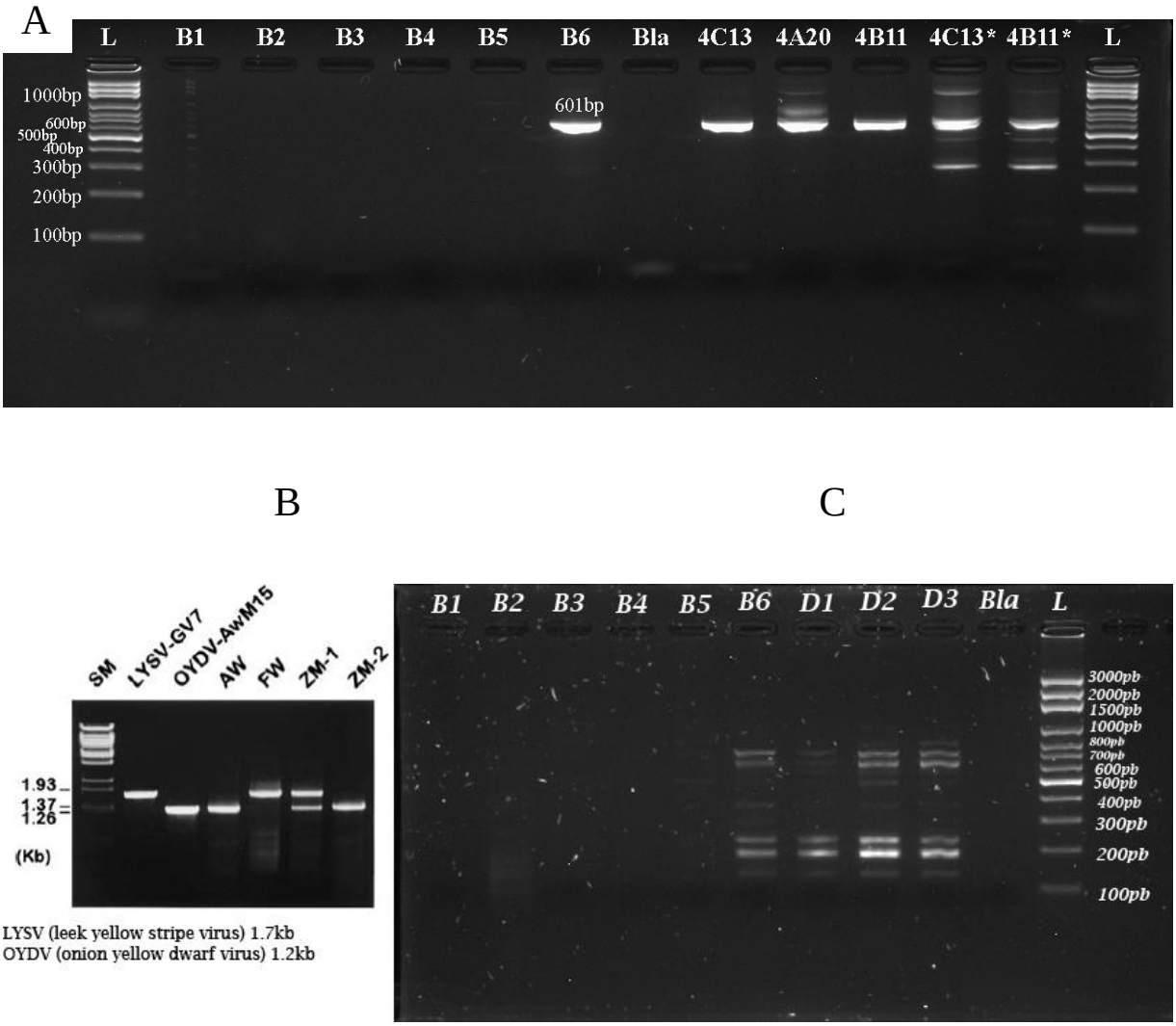
This is a restricted tissue (box) just under the base of the immature foliage leaves approximately 1-2mm thick (Ayabe & Sumi, 1998). A photograph of the stem disc in a position similar to this depiction can be found in Fig 8A.

Figure 3: Agarose gel electrophoresis of multiplex RT-PCR products from 12 shoot extraction garlic RNA samples.



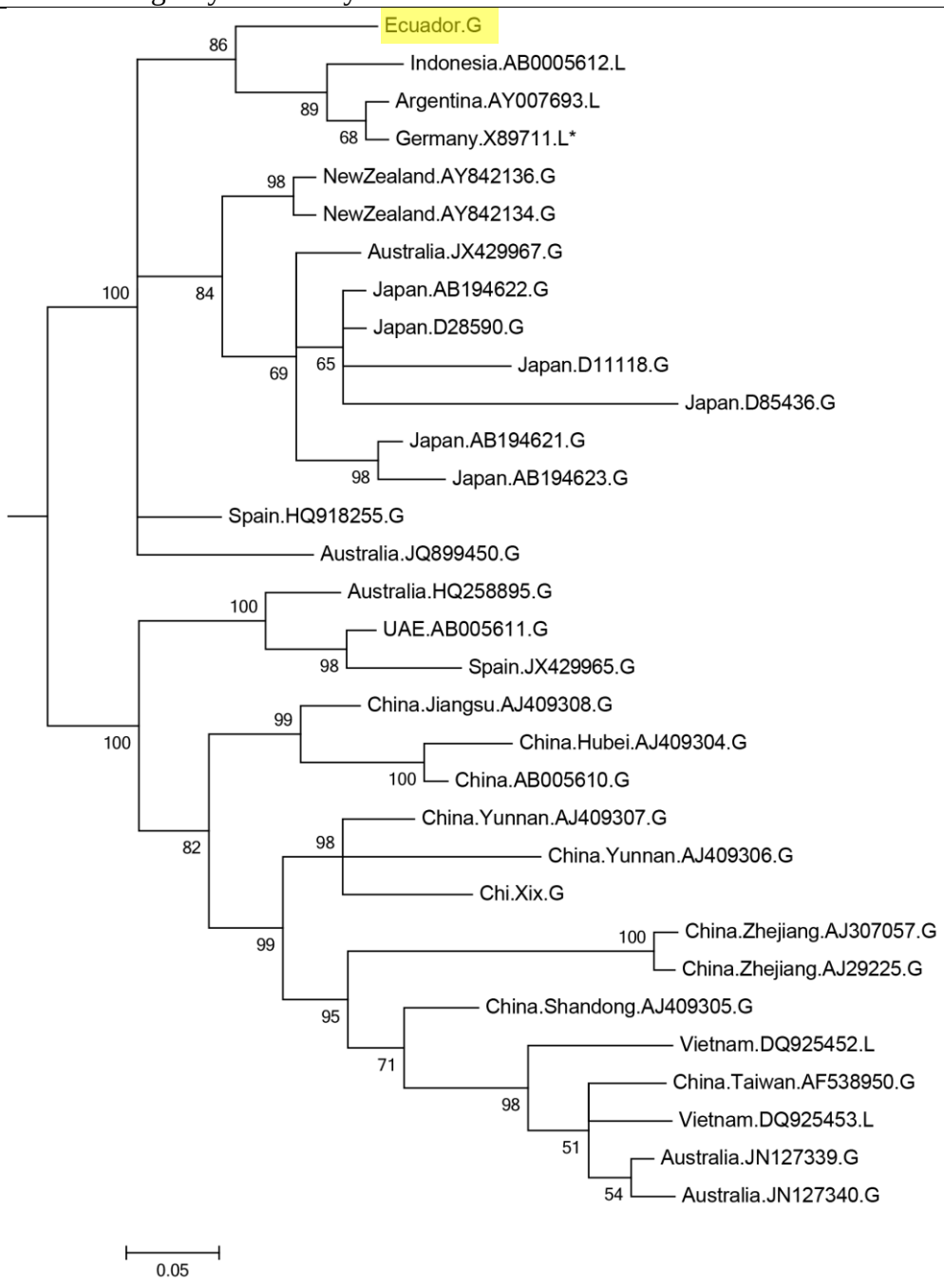
The electrophoresis was run for 130 minutes in a 3% agarose gel at 80 V. 8µL of each multiplex RT-PCR product was loaded in each well of the gel. SLV expected amplicon size was 308 bp, OYDV expected amplicon size was 290 bp and LYSV expected amplicon size was 191 bp. Sample AA_1 is negative for LYSV. Samples E_6, B_4, Y_9, AA_{13} and C_5 were SLV-negative. L: 100bp TrackIt DNA Ladder Invitrogen™

Figure 4: Agarose gel electrophoresis of RT-PCR products using primers O2 and Pot in garlic and shallot samples



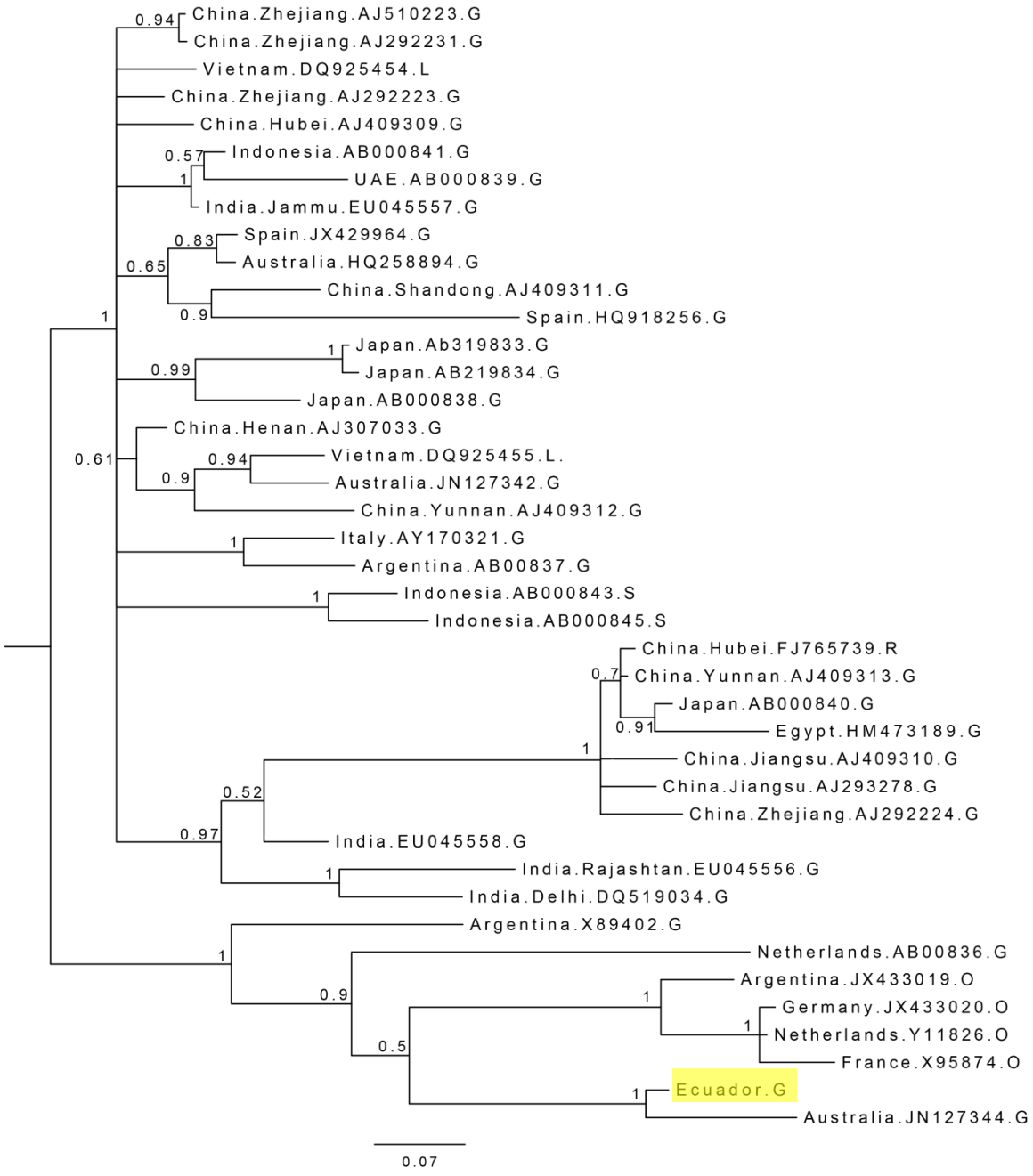
(A) OYDV RT-PCR products of 601 bp; B1-B5: RNA samples from garlic; B6, 4C13, 4A20, 4B11, 4C13*, 4B11*: RNA samples from shallot; Bla: blank (B) Expected amplicons for the Pot primer (Tsuneyoshi et al, 1998b) (C) Obtained RT-PCR products for the Pot primer; D1-D3: RNA samples from shallot. L: 100bp ladder Axygen™ *: duplicated RNA sample retrotranscribed in a separate reaction from the rest of the samples.

Figure 5: Phylogenetic consensus tree of the 3' untranslated region (c. 192 nucleotides) of LYSV obtained using Bayesian analysis



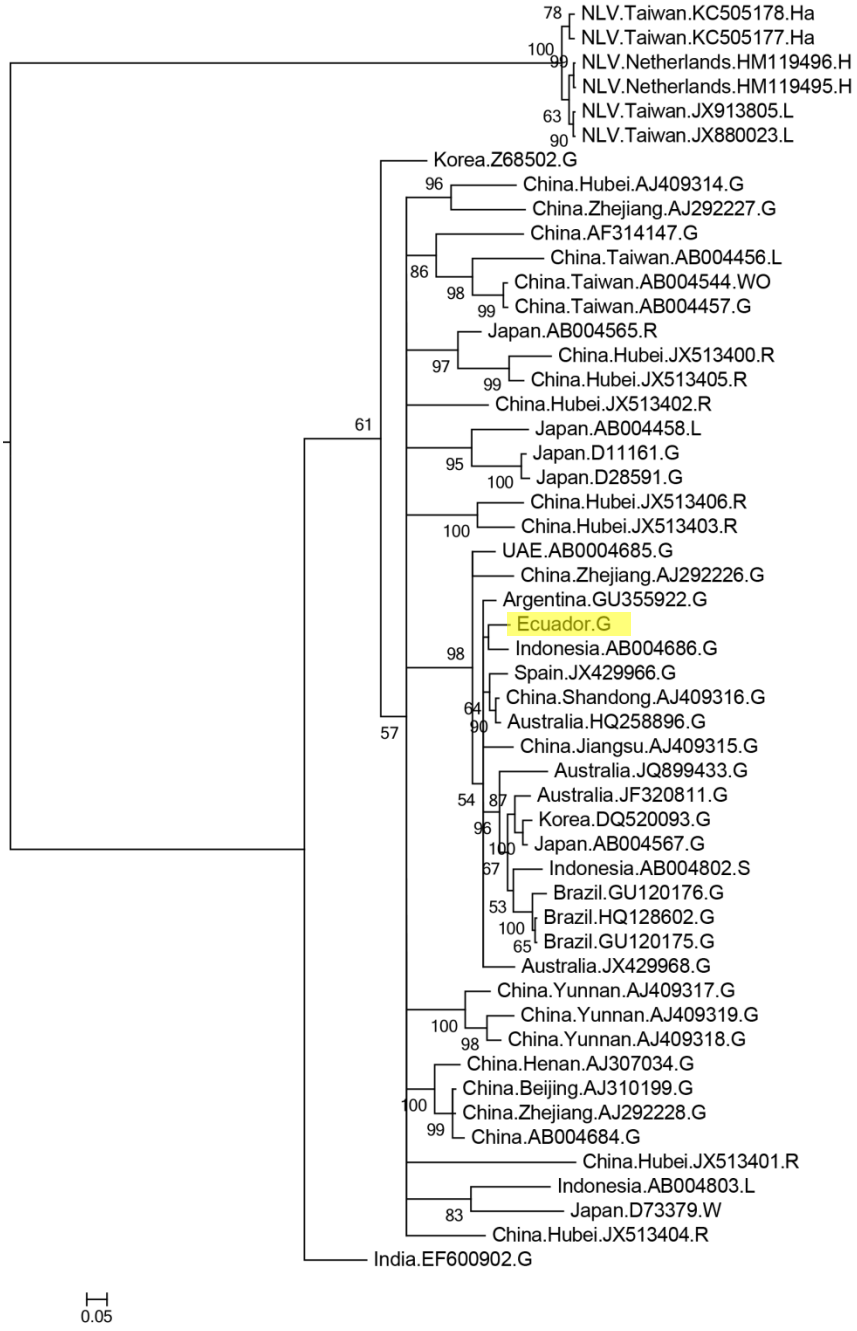
The nucleotide substitution model used for the analysis was K80 (Kimura, 1980) with gamma-shaped distribution of rates across sites. The tree is midpoint rooted (sequences evolve at the same rate). The values at the nodes indicate the posterior probability for the clade, branches with values of less than 50 are collapsed. G: garlic; L: leek; UAE: United Arab Emirates. *geographic location not certain

Figure 6: Phylogenetic consensus tree of the C-terminal region of the coat protein gene and the 3' untranslated region (c. 302 nucleotides) of OYDV obtained using Bayesian analysis



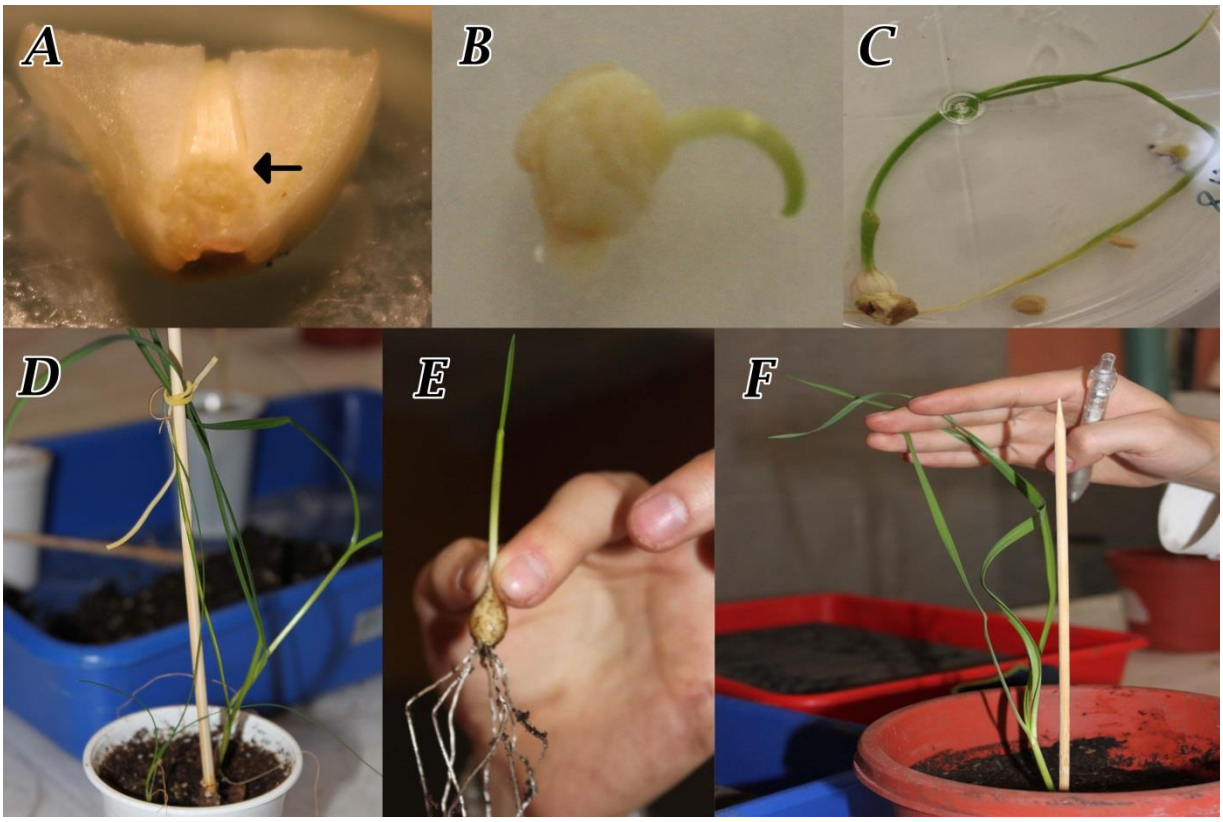
The nucleotide substitution model used for the coding region was K80 (Kimura, 1980) with gamma-shaped distribution of rates across sites, and for the non-coding region SYM (Zharkikh, 1994) with gamma-shaped distribution of rates across sites was used. The tree is midpoint rooted (sequences evolve at the same rate). The values at the nodes indicate the posterior probability for the clade, branches with values of less than 50 are collapsed. G: garlic; L: leek; O: onion; R: rakkyo; S: shallot; UAE: United Arab Emirates.

Figure 7: Phylogenetic consensus tree of the C-terminal region of the coat protein gene (c. 310 nucleotides) of SLV obtained using Bayesian analysis



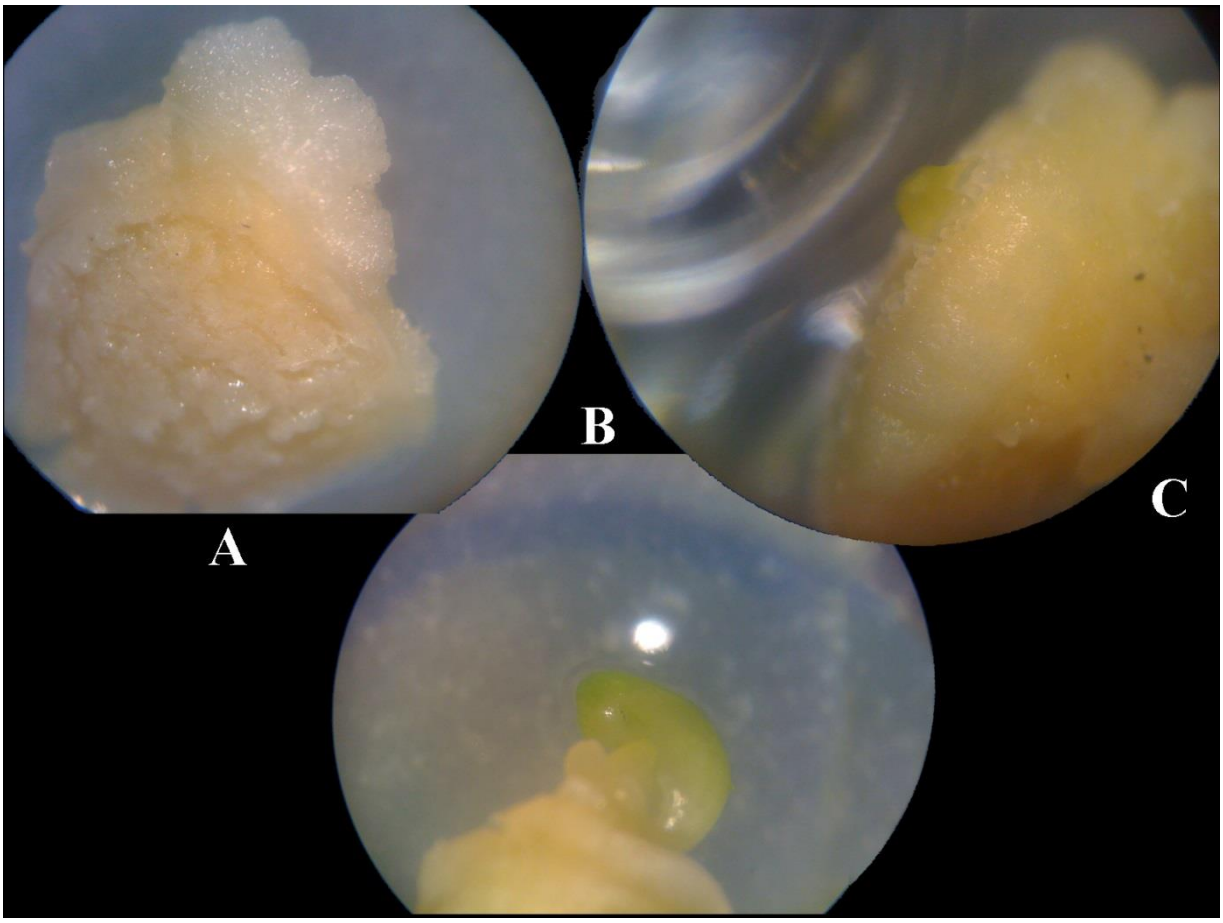
The nucleotide substitution model used for the analysis was GTR (Tavaré, 1986) with gamma-shaped distribution of rates across sites plus invariant sites. The tree is rooted in the nerine latent virus (NLV) clade (same as midpoint rooting). The values at the nodes indicate the posterior probability for the clade, branches with values of less than 50 are collapsed. G: garlic; H: *Hippeastrum*; Ha: *Haemanthus* (blood lily); L: leek; R: rakkyo; W: *Allium wakegi*; WO: welsh onion UAE: United Arab Emirates.

Figure 8: Different stages of the garlic tissue culture methods employed in this study



(A) Garlic clove half with the stem disc located at the arrow's tip (B) stem disc half cultured for 2 weeks with a 1 cm shoot, there was no apical disruption and only 1 shoot was generated. (C) Stem disc half cultured for 6 weeks with a bulblet and 2 leaves formed, this is from the direc bulblet formation procedure. (D) Garlic plantlet transferred to soil showing elongated leaves tightened to a stick because they were not strong enough to sustain themselves and fell to the ground otherwise (E) Garlic bulblet germinated in soil. (F) Garlic plantlet developed from a bulblet harvested from a plant previously transferred to soil.

Figure 9: Stereomicroscopic view of garlic stem discs at 20x magnification

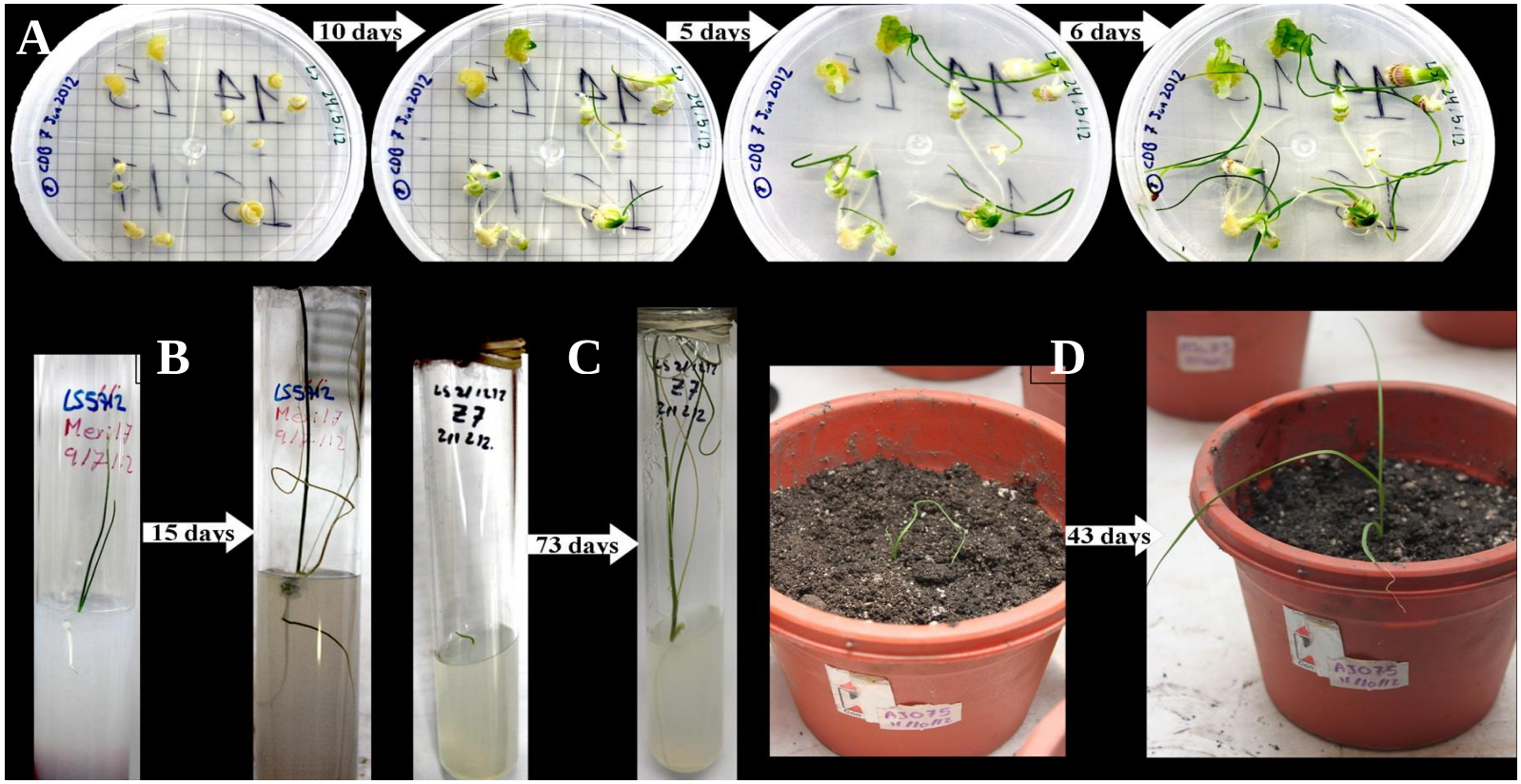


(A) Stem disc cultured on MS media after 20 days of culture. A callus can be seen at the top-right of the stem disc. (B) Stem disc cultured on LS media after 16 days of culture. A dome structure can be observed appearing on the surface of the stem disc. (C) Stem disc cultured on LS media after 20 days of culture. A developing shoot is clearly visible on top of the stem disc.

Figure 10: Garlic stem disc half with multiple shoots

6 shoots are seen developing from the top of a stem disc half after 14 days of culture in LS media (Appendix B). After shoot extraction, more shoots emerged from the same stem disc. The multiple shoot appearance seen could only be possible thanks to the disruption of apical dominance.

Figure 11: Chronology of the shoot extraction process from the stem disc to the planting in soil



The complete shoot extraction procedure used in this study starting from stem discs and ending in a potted plant is illustrated in this image. The shoot yields were highly dependent on the cutting technique of discs and shoots. The time required for the development of plants between pictures is shown inside the white arrows. **A:** development of shoots from stem discs. After shoots were extracted, stem discs were transferred to fresh media and yielded another batch of shoots. **B:** time required for the elongation and root development of a shoot extracted at 6 cm. **C:** time required for the elongation and root development of a shoot extracted at ~1 cm. **D:** plantlet from the shoot extraction procedure development with added fertilizer

Figure 12: Garlic stem discs that were left to form a bulblet without shoot extraction

Stem discs after 7 weeks of culture in LS media (Appendix B). Bulblets have started sprouting and are ready to be transferred to soil.

Figure 13: Garlic plant showing symptoms of viral disease

Severely dwarfed garlic plant from USFQ's fields infected with leek yellow stripe virus (LYSV), onion yellow dwarf virus (OYDV) and shallot latent virus (SLV). The leaf tips are necrotic due to viral infection. Chlorotic streaks and leaf deformation can be clearly seen.

14. APPENDICES

Appendix A: PCR protocols and primer sequences

Individual Amplifications.

A master mix was prepared as follows the individual amplification of each virus

Reagent	Initial Concentration	Volume per Reaction [µL]
PCR Buffer minus Mg	10X	1
$MgCl_2$	50 mM	0,3
Primer Mix (F+R)*	4 µM each primer	0,5
dNTP Mix	10 mM	0,3
Taq DNA Polymerase	5 U μL^{-1}	0,2
H_2O	n/a	to 10
cDNA		1

*Primer mix consisted of a solution of forward and reverse primers at a concentration of 4µM. A primer mix was prepared for SLV, OYDV and LYSV

Thermocycler program for individual PCRs:

Step	Temperature [ºC]		Time	Cycles
Initial denaturing	**LYSV, SLV**	94	5 min	
	OYDV	95		
Denaturing	**LYSV, SLV**	94	45 sec	30
	OYDV	95	1 min	
Annealing	**LYSV**	60	30 sec	
	OYDV	63	30 sec	
	SLV	53	20 sec	
Extension	72		1 min	
Final Extension	72		10 min	

Triplex-PCR.

The master mix for triplex-PCR was prepared as follows:

Reagent	Initial Concentration	Volume per Reaction [µL]
PCR Buffer minus Mg	10X	2
$MgCl_2$	50 mM	0,7
Primer Master Mix*	4 µM	0,5
dNTP Mix	10 mM	0,4
Taq DNA Polymerase	5 U μL^{-1}	0,2
H_2O		to 10
cDNA		1

* Primer master mix consisted of a solution of forward and reverse primers of LYSV, OYDV and SLV at a concentration of 4µM for each primer.

Thermocycler program for triplex PCR:

Step	Temperature [ºC]	Time	Cycles
Initial denaturing	95	5 min	
Denaturing	95	1 min	30
Annealing	57	20 sec	
Extension	72	1 min	
Final Extension	72	10 min	

Primer sequences.

LYSV and OYDV primer sequences were modified or degenerated (underlined nucleotides) from Sumi et al. (2001) according to sequence alignments of all sequences available for the respective locus in GenBank to date. SLV primer sequence was taken from Majumder et al. (2008). Every primer sequence was synthesized through Invitrogen's Custom Primers service.

LYSV:

L-F: 5'- AAG**R**GTCAACACTTGGTTTG -3'

L-R: 5'- GGTCTCAATCCTAGCTAGTC -3'

OYDV:

O3-F: 5'- GAAGC**A**CA**Y**ATGCAAATGAAG -3'

O3-R: 5'- **Y**GCCACA**R**CTAGTGGTACAC -3'

SLV:

S-F: 5'- GTGGTNTGGAATTAC -3'

S-R: 5'- CAACATCGATTYTCTC -3'

The following primers sequences did not yield the expected amplification products in garlic:

OYDV:

O1-F: 5’- ATAGCAGAAACAGCTCTTA -3’

O1-R: 5’- GTCTCYGTAATTCACGC -3’

(Majumder et al., 2008)

O2-F: 5’- CGAAGCAAATTGCCAAGCAG -3’

O2-R: 5’- CGATTACGTGCCCCTCTAAC -3’

(Mahmoud et al., 2007)

Potyvirus (OYDV+LYSV):

Pot-F: 5’- GCTATGGTCGAAGCATGGG -3’

Pot-R: 5’- ACCGATTCAACTGGAAGAATTCGCGG -3’

(Tsuneyoshi et al., 1998b)

Appendix B: Linsmaier and Skoog (LS) Medium

Macronutrients	mg L^{-1}
NH_4NO_3	1650.0
KNO_3	1900.0
$CaCl_2 \cdot 2H_2O$	440.0
$MgSO_4 \cdot 7H_2O$	370.0
KH_2PO_4	170.0

Micronutrients	mg L^{-1}
H_3BO_3	6.2
$MnSO_4 \cdot H_2O$	22.3
$ZnSO_4 \cdot 4H_2O$	8.6
KI	0.83
$Na_2MoO_4 \cdot 2H_2O$	0.25
$CoCl_2 \cdot 6H_2O$	0.025
$CuSO_4 \cdot 5H_2O$	0.025

Organics	mg L^{-1}
Thiamine HCl	0.4
Mio Inositol	100.0
Sucrose	30
Agar	10

Iron Chelate	mg L^{-1}
Na_2EDTA	37.3
$FeSO_4 \cdot 7H_2O$	27.8

pH adjusted to 5.6 (Linsmaier & Skoog, 1965).

Appendix C: RNA Extraction Protocol

Sample Collection.

- Fresh young leaves were cut and stored at 4ºC until reaching the laboratory.
- The leaf primordium was used when RNA was extracted from cloves.
- Samples were washed with tap water before starting extraction.

Extraction.

The whole procedure was done maintaining samples at 4ºC, even during centrifugation.

1. *Homogenization*

   1,5mL tubes where filled with 1.2 mL of Trizol Reagent (Invitrogen) and weighted. Plant tissue was homogenized in a mortar after freezing it with liquid nitrogen and 60-120 mg of pulverized tissue was added to the tubes. Samples were incubated in ice for 15 min to permit the complete dissociation of nucleoprotein complexes and insoluble material was removed from the homogenate by centrifugation at 12000rpm for 15min. Supernatant was transferred to a fresh tube.

2. *Phase Separation*

   240 µL of chloroform was added and the tubes where shook vigorously by hand for 15 seconds and incubated in ice for 3 min. Following centrifugation at 12000 rpm for 15 min, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase which contains the RNA. 700 µL of the aqueous phase were transferred to a new tube.

3. *RNA Precipitation*

   300 μL of citrate buffer was added and the tubes were homogenized by hand, next 500 μL of isopropyl alcohol was added to precipitate the RNA from the aqueous phase and the tubes were incubated for 10 min. Samples were centrifuged at 12000 rpm for 10 min and a pellet formed on the side and bottom of the tube, the supernatant was discarded.

4. *RNA Wash*

   1.2 mL of 75% EtOH were added to wash the RNA and after being vigorously shaken, samples were centrifuged at 7500 rpm for 5 min. Supernatant was discarded and the RNA pellet was dried for a maximum of 10 min under laminar hood flow.

5. *RNA Resuspension*

   50 μL of DEPC-treated water were added to resuspend the RNA and a final centrifugation at 12000 rpm for 20 min was done to eliminate undissolved residues.

Appendix D: First-Strand cDNA Synthesis Protocol

An initial master mix without RNA was prepared as follows:

Reagent	Initial Concentration	Volume per Reaction [µL]
DEPC-treated H_2O		11.9
dNTP Mix	10 mM	1
Not I-d$(T)_{18}$ primer*	4 µM	1
RNA	30-1000 ng μL^{-1}	1

* (GE Healthcare, 2008).

RNA was added to a final volume of 14.9 µL and the mix was denatured for 5 min at 65ºC followed by incubation in ice for 1 min. 5.1 µL from a second master mix was added to the reaction tubes.

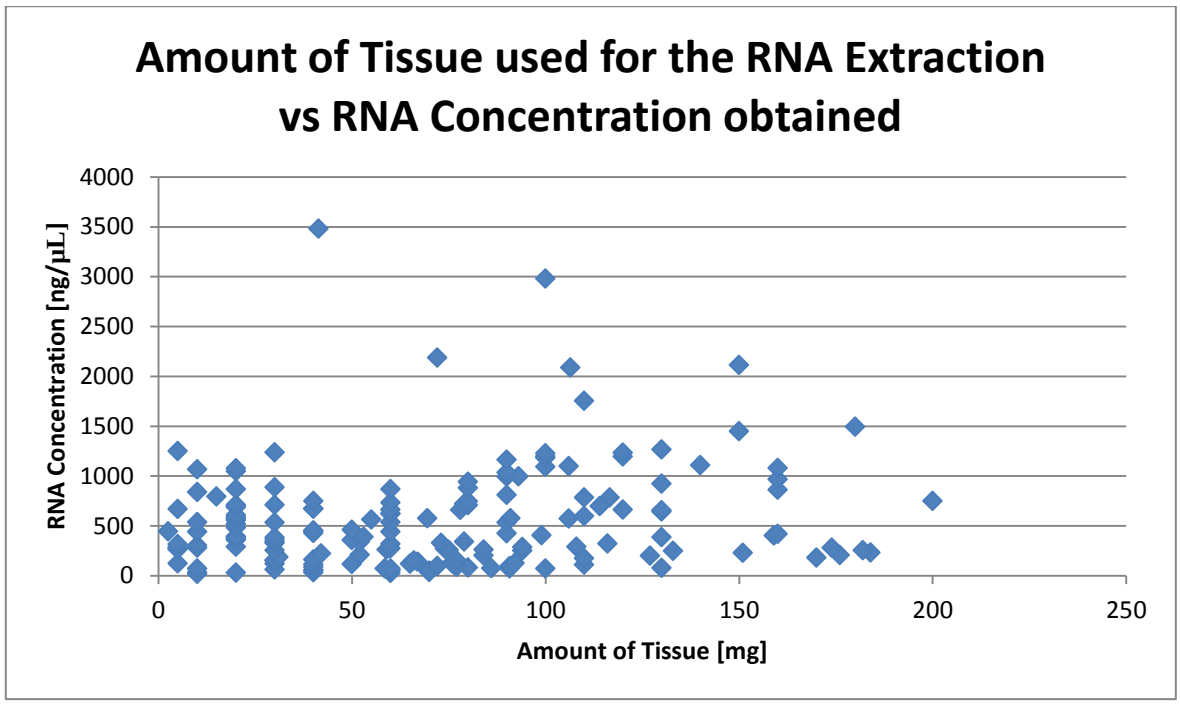
Reagent	Initial Concentration	Volume per Reaction [µL]
First-Strand Buffer	5X	4
DTT	0.1 M	1
SuperScript III RT	200 U μL^{-1}	1

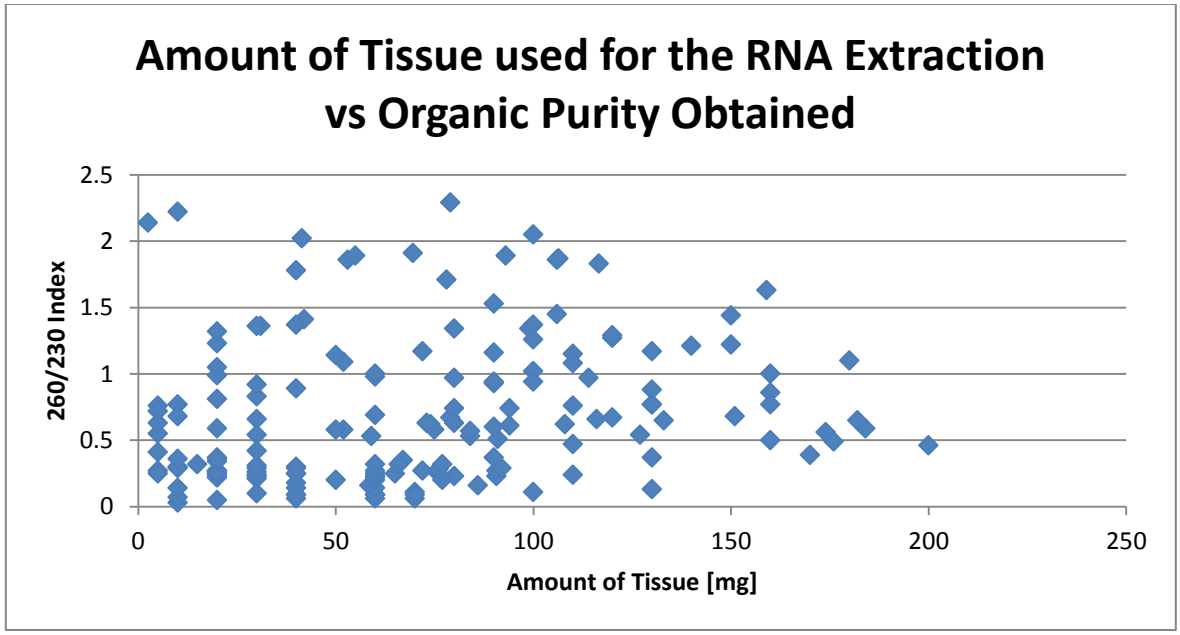
The final 20 µL reaction mixture was incubated at 37ºC for 1 hour and the reverse transcriptase was inactivated by incubation at 70ºC for 15 min.

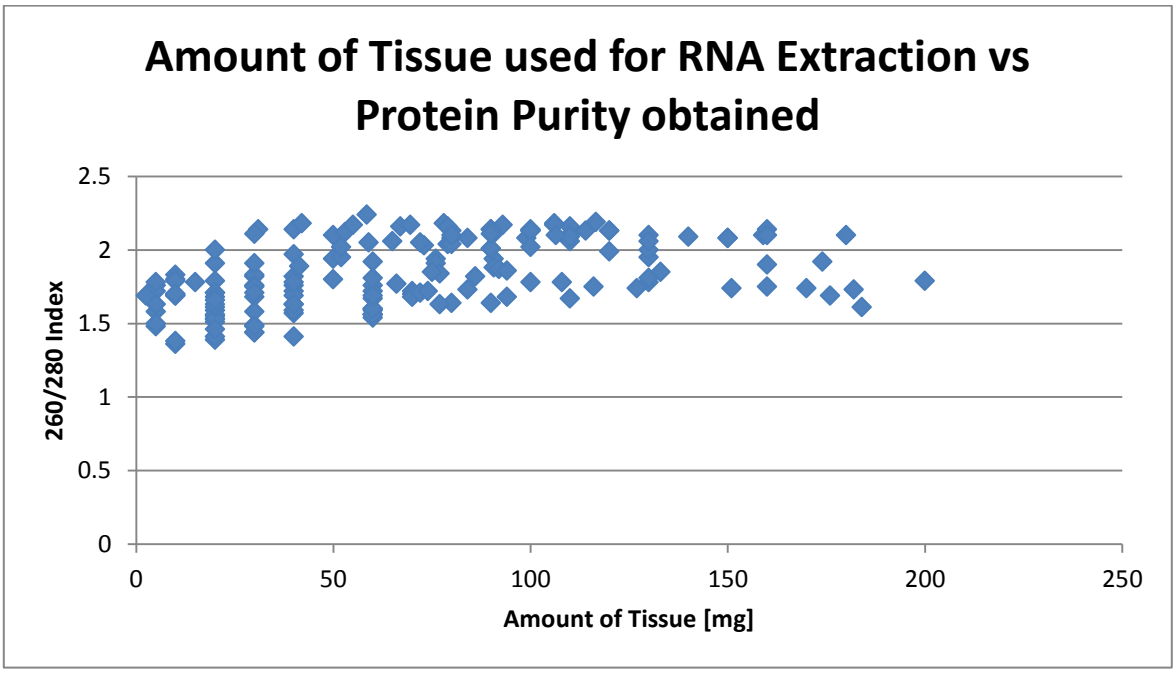
Not I-d$(T)_{18}$ primer sequence:

5’- AAC TGG AAG AAT TCG CGG CCG CAG GAATTTTTTTTTTTTTTTTT -3’

Appendix E: Scatter Plots of Protein Purity, Organic Purity and RNA Concentration obtained from the RNA Extraction of vs. the Amount of Tissue used for RNA Extraction







No curve assayed in excel fitted any of the scatter plots with an R^2 value of more than 0.5. For the correlation coefficients tested and their respective p-values see Table 1. Protein purity and organic purity values were obtained from the 260/280 index and the 260/230 index given by NanoDrop 1000 (Thermo Scientific). RNA concentration was similarly obtained from NanoDrop 1000 quantification. The amount of tissue used for the RNA extraction refers to the weight increase of Eppendorf™ tubes containing 1.2 mL Trizol Reagent (Invitrogen) after homogenized vegetal tissue was added to each tube.

Appendix F: Consensus Sequences

LYSV.

5’- AAGAGTCAACACTTGGTTTGAGGTTGATCGTCTGAAGATCTATCCGG
AGTACGGTTTTGTAAAGAAGGTGAATTCAGGTTTTAAGGATAGTAAGTCTGAAGC
CATAATGGTGTATAAGCAGCCTGAATCTTGATAACCCACCTTTACCCTACGATGGA
GTGCTACTTTAAAGACTAGCTAGGATTGAGACC -3’

OYDV.

5’- GAAGCACATATGCAAATGAAGGCGGCAGCGATTAGAGGGGCAACTAATCGT
TTGTTTGGCTTGGATGGTAACGTCAACACGACCGAAGAGGACACGGAAAGACACA
CAGCTGCCGATATTAATAAACATCAGCATACCTTACTTGGTATTAAGATGTAATTC
AGTTTGTGTGTCTTTAGTTTTATATATATATTTCTATATAAAAACGCACTTAGTATG
TATTCCTCTTTATTTCGTTTAACCTGACTTCGAGCAACGAAATGTGGTGTACCACT
AGCTGTGGCA -3’

SLV.

5’- GTGGTGTGGAATTACATGTTACTGAATGAGCAACCGCCCGCTAATTGGGAT
GCGAAGGGGTTCACCGAAAACACTAAATATGCTGCCTTTGATACTTTAGATGCCG
TGACTAACAAAGCCGCAATCCAACCACTCGAAGGTTTGATAAGGGCCCCGACTGA
TGCCGAACGCATTGCTTTCGCAACTCATAAGAAGTTAGCCCTTGCCAAGAATTCGC
AAAATTCCCGATACGCCAATACCTCTGCTGAGGTCACTGGAGGGTTCTTCGGATGT
CTGCCTAAAAACAATTTCAGAGAGAATCGATGTTG -3’