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Conservation of veteran trees within historical gardens (COVE): a case study applied to *Platanus orientalis* L. in central Italy

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ABSTRACT

Many veteran trees characterize the landscape of Mediterranean historical parks and gardens, representing unique values from ecological, cultural, social and historical points of view. The absence of natural regeneration is one of the main limits for their conservation. The main purpose of this research was to combine theoretical reasoning and practical solutions to maintain this rich tree heritage. A protocol based on an interdisciplinary approach was developed and applied to the plane trees (*Platanus* spp.) within the formal garden of Villa Lante of Bagnaia (Viterbo, Italy). The COVE analysis consisted of the following four phases: i) selection of veteran tree species after gathering and comparing historical information; ii) taxonomic identification based on morphological and molecular analyses; iii) tree inventory in GIS environment; and iv) vegetative propagation by woody cuttings and micropropagation.

The plane trees surveyed grow on three level terraces and significantly differ in size and phytosanitary conditions according to their topographic position. Molecular analyses indicated a genetic uniformity for the plane trees studied and their memberships to *P. orientalis* species. This evidence suggests that all specimens belong to the original planting dating back to 1576. All individuals showed a high susceptibility to the pathogen *Ceratocystis platani* even though so far there is no evidence of canker stain attack. Woody cuttings from the most vigorous individual showed 80% rooting rates. *In vitro* micropropagation using node culture approach provided healthy plants, maintaining the genetic fidelity respect to mother plant after three years of culture. The obtained results represent a successful example of integrated research for germplasm conservation and management of veteran trees within historical villas in Central Italy.

1. Introduction

Ancient and veteran trees aredefined as "trees of biological, aesthetic and cultural interest due to their age and size" (Lonsdale, 2013; Roggero, 2015; Fay and Butler (2017; Zapponi et al., 2017). Italy is particularly rich in this heritage with more than 1200 specimens uniformly distributed across the country belonging to 118 species (Zapponi et al., 2017). The most abundant species are *Quercus pubescens* Willd., *Fagus sylvatica* L., *Larix decidua* Mill., and *Platanus* spp. The criteria that rule the status of a monumental tree vary among European countries (Lisa, 2011). In Italy, during the last century, the awareness of their cultural and landscape values has increased leading to several laws and regulations. The first Italian law concerning protection of ancient trees dates back to 1939 (Law n. 1497, 29/06/1939), regarding them as "immovable things that have remarkable characteristics of natural beauty". This definition refers to the tree as a static element of the landscape. Recently, law n. 10 14/01/2013 gave the definition of monumental tree that includes single trees, tree lines and shrubs with a remarkable development, belonging to both native and non-native species. In addition, the Ministerial Decree 23/10/2014 identified seven criteria that should be met, jointly or alternatively (one criterion is sufficient), for the tree to be listed as monumental: significant age and size, peculiar shape, ecological value, botanic rarity, plant architecture, landscape value and historical-cultural-religious aspects (Zapponi et al., 2017). These elements recognize trees as living beings sustaining their eco-biological role. In Italy many Renaissance villas have a wide

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heritage of veteran trees. They add a forest character to monumental complexes also providing ecosystem services, (*e.g.* climate mitigation, habitat, cultural services, visitors wellbeing) and disservices, such as failure risk and underground root-artifacts interferences. Often these trees are the oldest in urban areas, and should be managed carefully, avoiding stress factors that compromise their longevity (Fay and Butler, 2017) or cause damages to structures and risk to human safety (Tomao et al., 2015). Furthermore very little is known about the provenance and genetic characteristics of different trees introduced into historical gardens for their symbolic value. This knowledge is very useful for choices regarding their conservation, perpetuation and propagation possibilities, even in other gardens.

This research aimed to develop a multidisciplinary methodology for the COnservation of VEteran trees within historical gardens (COVE) and their management. The secondary goal was the setting up of suitable methodologies to produce healthy trees from local germplasm for the replacement of damaged trees, and provide a GIS platform, upgradable with information periodically acquired, useful for site management. In this study, the model was applied to the historical garden of Renaissance Villa Lante della Rovere in Central Italy (henceforth Villa Lante) focusing on plane trees (*Platanus orientalis* L.).

2. Materials and methods

The general scheme of the COVE model is summarized in Fig. 1. It consists in a succession of four general steps leading to different outputs. In the present study COVE has been applied on *Platanus* spp., chosen as symbol tree, using methodologies described in detail in the following paragraphs. The study area was located within the monumental garden of Villa Lante (Viterbo, Central Italy, lat 42° 25′ 32″, long, 12° 09′ 18″; 460 m above sea level).

2.1. Selection of veteran tree species

The historical information about Villa Lante veteran trees come from the literature on the history of architecture concerning this monumental complex, including sources present at the Italian State Archives, Vatican Library, Ardenti Municipality Library-Viterbo, and Centro Studi Europeo della Tuscia-Bologna. The state of the garden was observed during a preliminary field trip in order to verify the presence of veteran trees, in particular plane trees, often present in Renaissance Villas in Central Italy.

2.2. Taxonomic identification

Species identification of plane trees at Villa Lante was assessed both by morphological and molecular analyses.

2.2.1. Morphological analysis

Veteran plane trees were identified on the base of leaves shape and bark patterns. In September 2014, for each tree, digital images of the bark and ten completely developed leaves, randomly collected from long branches formed at the end of the growing season (Vigouroux, 2007), were classified according to Pignatti (1987).

2.2.2. Molecular characterization

2.2.2.1. Sample collection. Three fully expanded and intact leaves of the same age were collected from a total of 40 plane trees, of which 23 from Villa Lante, two from Palazzo Farnese (Caprarola, Viterbo, Italy) and 15 accessions from an *ex situ* INRA collection maintained at Avignon (France), representing the three major *Platanus* species: *P. orientalis* L., *P. occidentalis* L. and *P. acerifolia* (Aiton) Willd. (Table 1). Once collected, leaves were immediately frozen in liquid nitrogen and stored at −80 °C until DNA extraction.

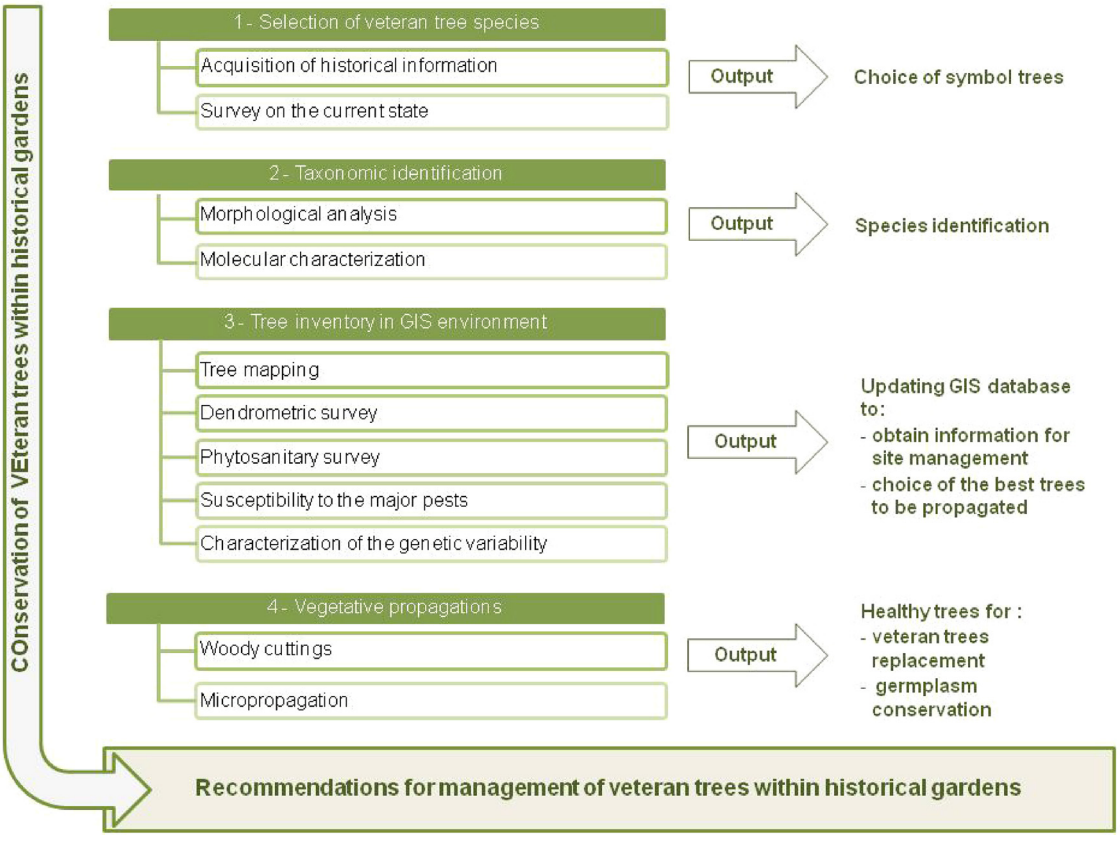

Fig. 1. COVE model flowchart: COnservation of VEteran trees within historical gardens.

Table 1
Species, individual codes, and origins of the plane trees included in the molecular analyses.

Species	N.	Code	Geographic origin
P. occidentalis	1	PocILL1*	Morgan County, Illinois, USA
	2	PocPB*	Athens, Georgia, USA
	3	PocG1*	Greenville, Mississipi, USA
	4	PocLAB*	Stoneville, Mississipi, USA
	5	PocM11*	Sikestone, Missouri, USA
P. acerifolia	6	PacBU*	Paris, France
	7	PacMUT*	Montpellier, France
	8	PacGB*	by Santamour (1972)
	9	PacPB*	by Panetsos (1984)
	10	PacVC*	by Vigouroux (2001)
P. orientalis	11	PorPB*	Macedonia Greece
	12	PorGPLK*	Platanakia, Greece
	13	PorSE2*	Samos Greece
	14	PorCRE1*	Xania, Crete Greece
	15	PorHAM*	Hamadan, Iran
	16	PorVL 1-23§	unknown
	17	PorCAP 1-2°	unknown

Place of collection: *INRA Avignon, France; §Villa Lante, Bagnaia (VT), Italy; ° Palazzo Farnese, Caprarola, Italy.

2.2.2.2. DNA extraction. Total genomic DNA was extracted using NucleoSpin® Plant II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The integrity and concentration of DNA were determined by 0.8 (w/v) agarose gels stained with ethidium bromide (0.001%) using known concentrations of unrestricted lambda DNA as control. All DNA samples were stored at -20 °C until use.

2.2.2.3. LEAFY gene analysis. Primer pair Un.For/Un.Rev was used to amplify specific fragments from both genomes of *P. orientalis* (about 700 bp) and *P. occidentalis* (about 800 bp), whereas primer pair Occ.For/Occ.Rev was used to verify the presence of a product of about 400 bp specific only for *P. occidentalis* (Pilotti et al., 2009; Table S1). PCR reactions were performed according to Pilotti et al. (2009). Samples (5 µl) of the amplification products were separated on 1.2% (w/v) agarose gels and visualized under UV radiation after staining with ethidium bromide (0.001%) and analyzed using the UVITEC Essential V6 Gel Imaging and Documentation System (Cleaver Scientific, Rugby, United Kingdom).

PCR products obtained with the primer pair Un.For/Un.Rev from four plants chosen as representative of the plane trees of Villa Lante were cloned into the pGEMT easy plasmid vector (Promega). Sequencing was performed on both strands by the ABI PRISM 377 capillary sequencer (PE Applied Biosystem) using an ABI Prism Dye Terminator sequencing kit (PE Applied Biosystem) and either vector- or sequence-specific primers. The specificity of the sequenced amplified fragments was checked by a BLAST search in the NCBI database.

2.3. Tree inventory in GIS environment

2.3.1. Tree mapping and dendrometric survey

During the spring 2015, a total of 23 plane trees were georeferenced and mesured to determine their main dendrometric parameters: i) diameter at breast height (DBH; cm), ii) total height (m), iii) crown base height (m) and iv) crown projection area (m^2), using a portable computerized station Field-Map - Antelope model® (IFER-MMS Ltd, Prague, Czech Republic). The tree volume and biomass was estimated according to Tabacchi et al. (2011).

2.3.2. Phytosanitary survey

All plane trees were visually inspected for the presence of Canker Stain Disease (CSD - bark lesions, wilting, chlorosis and microphyllia) and stem decays. Disease severity (S) was estimated by the equation $S = \Sigma(x_i n_i)/n$, in which x represented disease grade as in Table S2, while n_i represented the number of diseased plants on the i^{th} grade of the disease scale and n was the total number of diseased plants evaluated. Disease incidence ($I = \Sigma x/n$) was the proportion of diseased plants, which consisted of the number of diseased plants (x) divided by the total number evaluated (n) (Groth et al., 1999).

2.3.3. Susceptibility to Ceratocystis platani (Walter) Engelbr. & T.C. Harr

Inoculation tests were made according to Vettraino et al. (2011), lightly modified, with two representative isolates of *C. platani* (VT1 and VT2, isolated in Viterbo and maintained at the Mycological Collection, DIBAF, University of Tuscia, Italy) cultured on MEA (Malt Extract Agar) for 1 week prior to use. Twigs of approximately 4–9 mm diameter were taken from 5 healthy mature plants, chosen as representative of the plane trees at Villa Lante. Foliage was removed and the twigs were cut to approximately 25 cm in length. Following wiping with cotton wool soaked in 70% ethanol, a 3 mm diameter bark disc was removed from the central point of each twig using a cork borer and re-placed by an equal-sized disc taken from the margin of a freshly growing culture. Lesions were wrapped with moist gauze and sealed with Parafilm. Controls were inoculated with sterile MEA discs. Five twigs were inoculated per isolate or control. After inoculation, shoots were incubated in a ventilated chamber for 1 month at 21 °C and 100% relative humidity. After incubation, the length of bark necrosis was measured on each shoot. Re-isolation was attempted by transferring ten pieces of diseased tissues taken from the margin of each lesion to MEA at 20 °C, to confirm that the isolates inoculated were still present in the lesions.

2.3.4. Characterization of the genetic variability

The genetic variability among the plane trees of Villa Lante and their genetic relationships with representative *P. orientalis*, *P. occidentalis* and *P. acerifolia* accessions were analyzed by ISSR markers. DNA samples were amplified with 15 ISSR primers (Table S3) previously used for the determination of genetic stability of micropropagated plantlets of *P. acerifolia* (Huang et al., 2009) and for the genetic characterization of morphological variants of *P. occidentalis* cultivated in Korea (Lee et al., 2012). PCR reactions were performed in a total volume of 25 µl containing approximately 20 ng of genomic DNA, 0.5 µM of each primer and 12.5 µl of Hot Start PCR Master Mix, 2× (Biotechrabbit, Hennigsdorf, Germany). All reactions were carried out in a Eppendorf Thermal Cycler (Mastercycler Gradient) with the following parameters: initial denaturation at 95 °C for 4 min, 35 cycles of amplification, each at 94 °C for 1 min, 50–59 °C for 1 min (depending on the optimal annealing temperature of the different primer employed), 72 °C for 2 min, and a final extension at 72 °C for 7 min. Each ISSR PCR reaction was independently repeated two times to test the amplification reproducibility. Amplification products were separated on 1.5% (w/v) agarose gels and visualized as described previously.

2.4. Vegetative propagations

2.4.1. Woody cuttings

During winter 2015, epicormic branches (2–3 per plant, 1–2 m long, placed at 4–5 m from the soil) were collected on January from 11 veteran trees representative of all phytosanitary classes. The distal and healthy parts of the branches were enveloped in plastic bags and maintained at + 4 °C. On February, one (10–12 cm) and two years (12–15 cm) branches (10 + 10 per plant) were cut from the collected material, treated with a fungicide (CAP WDG-Siapa®, 2 g L^{-1}) and planted in a greenhouse (overall 210 cuttings). The cuttings were inserted into a closed bench filled with a perlite substrate, equipped with a bottom heat system (20–23 °C). They were regularly watered with tap water 2 times per week. On March, the rooted cuttings were transplanted in plastic pots (3 L) in a mixture of sand (30%) and potting soil (70%) and evaluated for rooting and number of roots per plant.

Table 2
Concentration (mM) of the macroelements, Cl^- and Fe^{++} in the six *in vitro* substrates utilized for *P. orientalis* micropropagation, in the multiplication stage.

	MS	GD	WPM	KP	KOP	KROP
N_{tot}	60.01	36.95	14.70	60.01	17.86	14.70
NH_4^+	20.61	12.49	5.00	20.61	0.00	5.00
NO_3^-	39.40	24.46	9.70	39.40	17.86	9.70
K^+	20.05	12.96	12.61	20.05	10.66	12.62
Ca^{++}	2.99	1.04	3.00	2.99	7.22	3.00
Mg^{++}	1.50	0.14	1.50	1.50	1.50	1.50
PO_4^{--}	1.25	2.20	1.25	1.25	1.25	1.25
SO_4^-	1.63	0.15	7.34	1.83	1.83	7.51
Cl^-	6.00	0.87	1.31	6.55	6.55	1.88
Fe^{++}	0.10	0.10	0.10	0.20	0.20	0.20

2.4.2. Micropropagation

Various substrates (MS, GD, and WPM) were used in the past for micropropagation of plane trees, almost exclusively for *P. acerifolia* (Gjuleva and Atanasov, 1994; Arène et al., 2002; Grolli et al., 2004; Alegre et al., 2015). All these culture media were tested and compared with KP, KOP, and KROP, developed in the present study (for their detailed formulations, see Table 1 in Kuzminsky et al., submitted). The concentration of the macroelements, as well as chlorine and iron, of the six substrates is reported in Table 2. The substrates were supplemented with agar (Difco, 7 g L^{-1}) and adjusted to pH 5.8 before sterilization in autoclave at 120 °C for 20 min. Subcultures were done every 15 days. The material was maintained in a growth chamber at 23 °C and 16 h-photoperiod of 50 ± 5 µmol m^{-2} s^{-1} (PAR, cool-white fluorescent tubes). Night temperature was 21 °C. The different stages of micropropagation were carried out as follows.

2.4.2.1. Establishment of in vitro culture. On March 2015, epicormic branches of 8 plants belonging to the 0–1 phytosanitary classes were collected. Shoots of one year old were sterilized according to Grolli et al. (2004). Node explants (100 for each individual) were single cultured on KP substrate only, and after 30 days, the contaminated explants were discarded.

2.4.2.2. Multiplication. Healthy shoots (4–5) per substrate were deprived of apical tips and put in the multiplication media added with 6-benzylaminopurine (BAP) (0.5 mg L^{-1}), a cytochinin able to induce axillary shoots formation. After 1 month the multiplication rate were quantified. The clumps were divided at each subculture in order to obtain enough micropropagated shoots for rooting. Multiplication stage was carried out for 4 months.

2.4.2.3. Elongation and rooting. Before rooting the material were prepared for elongation reducing sucrose (20 g L^{-1}) and the BAP content (0.050 mg L^{-1}). The addition of 0.02 mg L^{-1} of Indole-3-butyric acid (IBA) was adopted to promote adventitious roots emission from the clumps.

2.4.2.4. Hardening. *In vitro* rooted material was planted in plastic pots (3 L) in a mixture of sand (30%) and potting soil (70%). The plants were covered with a transparent glass container for *post-vitro* acclimatization by gradually weaning them towards ambient relative humidity. After one month, the rooted plants were transferred to the greenhouse.

The genetic fidelity of 10 micropropagated plants after 3 years of *in vitro* cultivation was tested respect to mother plant, amplifying their DNA with the 15 ISSR primers previously described.

2.5. Data analysis

Only distinct, reproducible and well-resolved ISSR fragments ranging from 200 to 3 Kb were considered in the analysis. The DNA bands were scored either as present (1) or absent (0) for each of the ISSR markers within all the genotypes analyzed. Data were entered in a Microsoft EXCEL spreadsheet to create a binary matrix. Genetic similarity coefficient was estimated using the equation $2a/(2a + b + c)$ (Nei and Li, 1979); where a is the number of bands shared by genotypes i and j, b is the number of bands present in i and absent in j, c is the number of bands present in j and absent in i. The pairwise genetic distances for phylogenetic relationships among genotypes were calculated from the Nei and Li similarity coefficients as: 1- similarity. The corresponding distance matrix was used to construct a UPGMA dendrogram by MEGA 7 software (Kumar et al., 2016).

A further classification was made using STRUCTURE v. 2.3 software (Pritchard et al., 2000). In particular, STRUCTURE was used to assign individuals to different plane species and identify admixture individuals. The RECESSIVEALLELE = 1 algorithm for dominant markers was used, where 0 = recessive allele for each *locus*. The number of inferred groups was evaluated at values of K ranging from 2 to 10, a burn-in length of 50,000 followed by 10 runs at each value of K. Estimates were obtained under the admixture model using the correlated allele frequencies and the LocPrior algorithm, which incorporates population information and is appropriate for detecting weak population structure (Falush et al., 2007). The most likely number of clusters (K) was determined using the ΔK method, as well as by examining the plateau of the Ln Pr(X/K) (Evanno et al., 2005).

Statistical analysis was performed using Prism version 5.00 software (GraphPad Software, California, USA). Data of susceptibility to *C. platani*, number of roots per cutting for the group of plant with a value of rooting > 40%, multiplication and elongation rate of axillary shoots were submitted to one-way ANOVA and the means were compared by using Tukey's pairwise tests at a significance level of 0.05. Chi square test was conducted to check whether there were significant differences in rooting success of the different stem cuttings collected from different aged branches (Badji et al., 1991).

3. Results

3.1. Selection of veteran plane trees

3.1.1. Historical information and survey on the current state

The formal garden of Villa Lante is structured with terraces on three levels. The presence of plane trees, planted symmetrically with respect to the axis of the water line, from Palazzina Gambara and Palazzina Montalto (first terrace) to the top of the hill (third terrace), is referred since the XVI century (Frommel, 2005). In a letter dated 1576, sent by Cardinal Gambara to Ottavio Farnese (Duke of Parma and Piacenza and brother of cardinal Alessandro of Caprarola) the establishment of a formal garden with plane trees within the Villa is described (Varoli Piazza, 1990; Fagliari Zeni Buchicchio, 2005). This event was confirmed, in 1578, by the clergyman Fabio Arditio (Orbaan, 1920) and, in 1603, by an asset inventory of the property (Fagliari Zeni Buchicchio, 2005). In 1656, the Villa was inherited by the Lante della Rovere family and remained theirs, in perfect conditions, until 1933 (Frittelli, 1984). A long period of neglect, before and during World War II, damaged the Villa. Afterwards the Villa Lante Company (1954) and then the Italian Government (that purchased the monumental complex in 1973) took care of the property. At present, only 23 individuals survived compared to the 56 plane trees previously reported (Orbaan, 1920).

3.2. Taxonomic identification

3.2.1. Morphological results

All the plane trees showed *P. orientalis* typical leaf shape consisting in palmate, deeply lobed leaves with narrow central lobe and more teeth than *P. acerifolia*. The bark peeled off in flakes, rough looking (Pignatti, 1987). However, the presence of common traits between the hybrid species and *P. orientalis* does not allow precise species

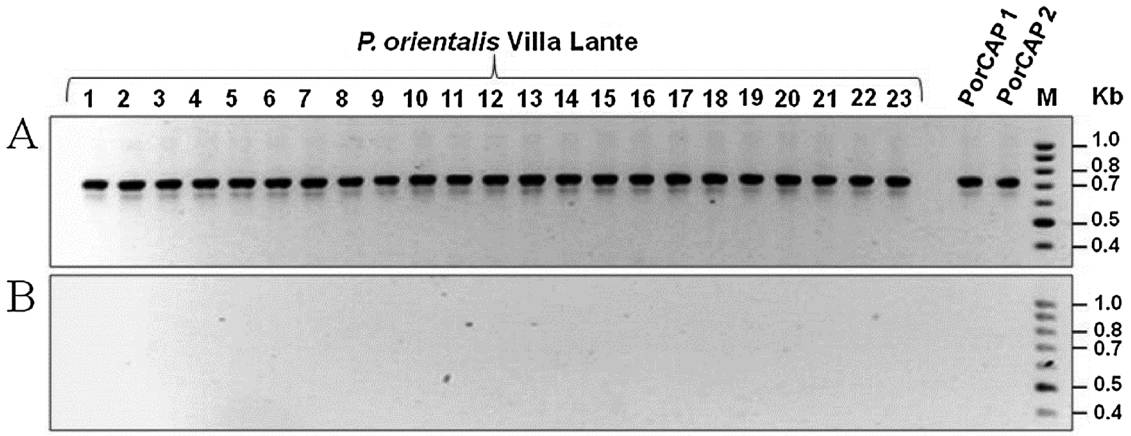

Fig. 2. Agarose gel electrophoresis of PCR products by primer pairs Un.For/Un.Rev (A) and Occ.For/Occ.Rev (B) of 23 plane trees of Villa Lante and the two plants of Palazzo Farnese, Caprarola.

identification, morphologically.

3.2.2. Molecular characterization

Amplification of the *LEAFY* gene with Un.For./Un.rev primers showed the presence of a single product of about 700 bp characteristic of *P. orientalis* for both the 23 plane trees of Villa Lante and PorCAP1 and PorCAP2, from Palazzo Farnese (Caprarola, Viterbo) (Fig. 2A). As expected, amplification with Occ.For/Occ.Rev primers did not result in any amplification product (Fig. 2B). The sequences of PCR products with Un.For./Un.Rev primers from the four plants chosen as representative of the plane trees of Villa Lante, were identical and showed high level of homology (identities comprised between 97–100%) with *LEAFY P. orientalis* sequences deposited in NCBI databases (*e.g.* AY706059.1, AY706058.1 and AY706060.1).

Using the Un.For./Un.rev primers, DNA fragments of expected size in *P. occidentalis* (about 800 bp) and *P. orientalis* (about 700 bp) accessions were obtained analyzing *P. occidentalis*, *P. orientalis* accessions used as references (Fig. 3A). Within the same species small differences in size of PCR products were detected both for *P. occidentalis* and *P. orientalis* accessions, indicating the presence of different alleles (Fig. 3A). Moreover, the accession PocM11 (lane 5 Fig. 3A) clearly showed the presence of two differently-sized bands representing different *P. occidentalis LEAFY* alleles, suggesting that this accession is heterozygous for the *LEAFY* gene. Electrophoretic pattern of primers Un.-based PCR showed the presence of DNA fragments from both genomes of *P. orientalis* and *P. occidentalis* in three of the five *P. acerifolia* accessions analyzed (lanes 6, 9 and 10, fig.3A), with the PacBU accession (lane 6) possessing different *P. orientalis* and *P. occidentalis* alleles in comparison to PacGB and PacPB (lanes 9 and 10). An unexpected result was obtained for the *P. acerifolia* accessions PacBU and PacMUT, for which, in addition to the amplification product of the *LEAFY* gene for the *P. orientalis* genome (lanes 7 and 8, Fig. 3A), no specific product was detected for the *P. occidentalis* genome (lanes 7 and 8, Fig. 3A). Amplification products obtained using the primer pair Occ.For/Occ.Rev confirmed the results of primers Un.-based PCR with regard to the presence of *P. occidentalis LEAFY* homoeologues in all the accessions analyzed (Fig. 3B), including the two *P. acerifolia* accessions PacBU and PacMUT (lanes 7 and 8 in Fig. 3B).

3.3. Tree inventory in GIS environment

3.3.1. Tree mapping and dendrometric survey

The map of plane trees within the three terraces of the formal garden is showed in Fig. 4. Their main dendrometric attributes are

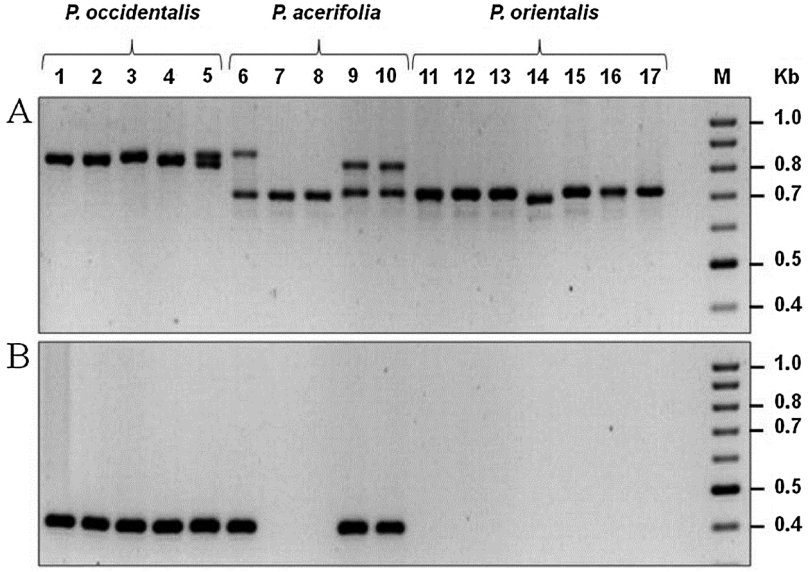

Fig. 3. Agarose gel electrophoresis of PCR products by primer pairs Un.For/Un.Rev (A) and Occ.For/Occ.Rev (B) of accessions used as references (N. 1–15 in Table 1) and PorVL1 from Villa Lante (lane 16) and PorCAP1 from Palazzo Farnese, Caprarola (lane 17).

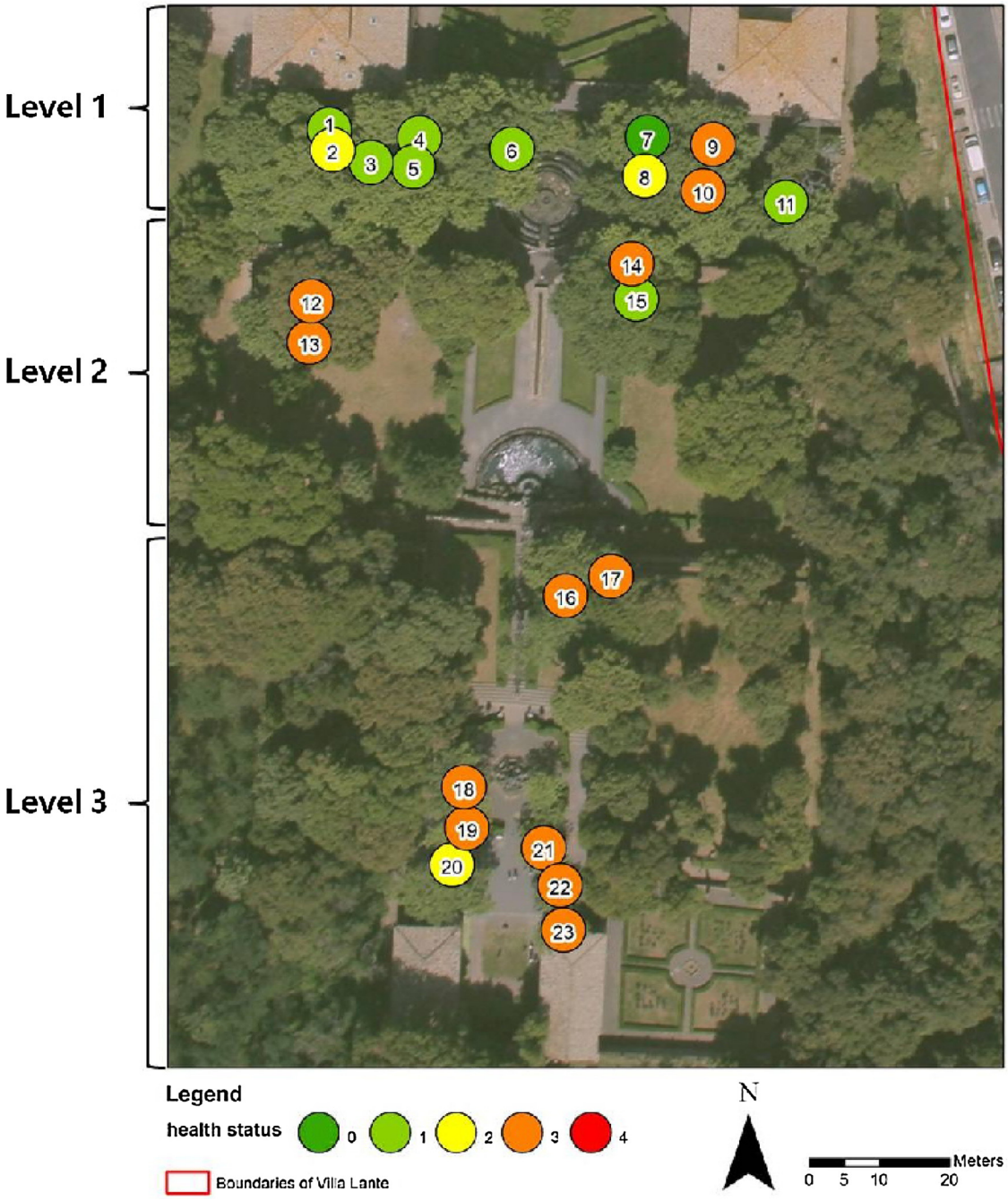

Fig. 4. Villa Lante della Rovere, Viterbo. Map of plane trees in different health status, within the three terraces of the formal garden, obtained adopting five classes of disease severity: from 0 (healthy tree) to 4 (dead tree).

Table 3
Dendrometric data of plane trees.

Dendrometric data	Mean	Range	Coefficient of variation
Diameter at the breast height	115.04 cm	47.00-171.00	0.21
Total height	16.58 m	5.80-24.60	0.34
Crown base height	5.09 m	2.80-8.50	0.30
Crown projection area	86.50 m^2	8.88-353.14	0.86
Volume	7.58 m^3	0.91-19.00	0.60
Biomass	2032.80 kg	311.49-4843.38	0.55

reported in Table 3. Plane trees are characterized by a mean DBH of 115 cm (SD = 24.4 cm) and a total height of 16.58 m (SD = 5.59 m). Around 75% (18/23) of the trees have a DBH greater than 100 cm, reaching in one case 171 cm. Nearly 40% (9/23) of the trees were higher than 20 m, while two are lower than 10 m. The mean volume is high, equal to 7.58 m^3 (SD = 4.57 m^3), with a maximum value of 19 m^3. The crown projection area is characterized by the greater variability (CV = 0.86) ranging from 8 m^2 to 353 m^2 (mean = 84.5 m^2; SD = 74.59 m^2).

Even if the diameter variability is not very high (CV around 0.2), there are some individuals with exceptional DBH values, reaching in one case 171 cm. For this reason, and given the considerable importance from the cultural and historical point of view, they could be classifiable as monumental trees, according to the 39/2002 regional

law and to the 10/2013 national law. On the other hand, the mean total height (16.5 m) is lower than that usually characterizing large planes. This result is probably due to continuous pruning with the aim of reducing branch failure risk, which is a very relevant issue to be considered in the management of veteran trees within intensely frequented historical sites (Tomao et al., 2015). Furthermore, it is important to point out that the trees considered are close to targets such as buildings or artifacts, which could be seriously damaged in case of tree failure,in particular, in the first terrace, where the larger plane trees grow.

3.3.2. Phytosanitary survey

Plane trees were assigned to four of the five identified health classes (Fig. 4). About 65% of the trees observed were classified from level 2 to level 3 severity class. None were considered class 4 (dead tree). Most of the trees showed symptoms of decline, consisting of yellowing and low crown density. The incidence index of the area was 0.96, with severity of 0.74 and 1.13 in the first terrace and the upper part of the garden, respectively. No lesions and bark swelling or cracks, that could be associated to CSD, were detected on the trunk or branches. Severe decay was detected in about 65% of the inspected trees, with cavities reaching the diameter of the trunk. The greater amount of decay of sampled trees was in the upper part of the garden (58%).

3.3.3. Susceptibility to Ceratocystis platani

Both isolates of *C. platani* used in pathogenicity tests caused lesions on freshly cut twigs of plane trees of Villa Lante. There were no significant differences ($P > 0.05$) in aggressiveness between the isolates, thus data were combined. Necrotic lesions averaged 85.3 ± 0.4 mm in length and were significantly longer (T-test $P = 0.005$) than those on control twigs. *C. platani* was successfully re-isolated from lesions, thus fulfilling Koch's postulates. Random re-isolations demonstrated that the necrosis was caused by the inoculated isolates; hence, no *C. platani* was isolated from the control twigs.

3.3.4. Characterization of the genetic variability

On the base of the ISSR analysis (Table 4), a total of 162 bands, ranging from 200 bp to 3 kb in size were generated across the plant material, with an average of 10.8 per primer. The number of bands varied from 4 (ISSR28) to 15 (ISSRs 13-20-38-46). Three primers (ISSRs28-43–44) were not polymorphic, whileUBC879 and ISSR46 produced the lowest (4) and the highest (14) number of polymorphic bands, respectively. Overall a total of 115 polymorphic bands were produced (71% polymorphism), with the lowest value of polymorphism for *P. occidentalis* (32.43%) and the highest for *P. acerifolia* (43.38%).

Concerning the 23 specimens of Villa Lante, a remarkable genetic similarity, with an identical ISSR amplification pattern for a total of 110 bands (loci), was detected (Fig. S1). It is worth noting that also PorCAP1 and PorCAP2, the plane trees present at the garden of Palazzo Farnese at Caprarola, were characterized by identical ISSR patterns with a total of 105 bands (loci), slightly different from those detected at Villa Lante (Fig. S1).

In Figure S2, the polymorphic patterns of 8 out of 15 ISSR primers from different accessions of *P. occidentalis*, *P. acerifolia* and *P orientalis*, including PorVL1 and PorCAP1, is reported.

A similarity matrix based on Nei and Li coefficients was constructed to estimate the level of relatedness among the different *Platanus* genotypes used in the present study (Table S4).

Pairwise estimates of similarity ranged from 0.616 (between PorVL1–23 and PocPB) to 0.949 (between PorVL1–23 and PoCAP1–2). *P. acerifolia* and *P. orientalis* were the closest species with the highest mean similarity index (0.793), followed by *P. occidentalis* and *P. acerifolia* (0.757) and *P. occidentalis* and *P. orientalis* (0.658). A dendrogram was constructed based on Nei and Li's dissimilarity coefficients using the UPGMA algorithm. All the genotypes were grouped in three clusters corresponding to *P. orientalis*, *P. acerifolia* and *P. occidentalis* groups (Fig. 5A). The cluster formed by *P. occidentalis* was clearly distinct from the others. Plane trees from Villa Lante and Palazzo Farnese at Caprarola clustered together with *P. orientalis* accessions from INRA, confirming the results of amplification of the *LEAFY* gene (Fig. 5A).

The genetic structure and the likely number of genetic groups (K) within the whole collection of *Platanus* genotypes analyzed were evaluated by STRUCTURE. Three putative genetic pools corresponding to the different *Platanus* species were detected using the method proposed by Evanno et al. (2005) (Fig. 5B). Moreover, according to the UPGMA analysis plane trees from Villa Lante and Palazzo Farnese at Caprarola were both assigned to the group containing the *P. orientalis* accessions from INRA. Admixture levels were detected only in individuals belonging to the *P. acerifolia* group, confirming their hybrid origin (Fig. 5B).

Table 4
Size range, number of total and polymorphic amplification products, and percentage of polymorphism for the primers used in this study in all 17 plane tree genotypes analyzed and in *P. occidentalis* (5), *P. acerifolia* (5) and *P. orientalis* (7) genotypes considered separately.

Primer	Size Range (Kb)	All genotypes			*P. occidentalis*			*P. acerifolia*			*P. orientalis*		
		Tot[a]	Pol[b]	Pol[c] (%)	Tot[a]	Pol[b]	Pol[c](%)	Tot[a]	Pol[b]	Pol[c](%)	Tot[a]	Pol[b]	Pol[c](%)
ISSR12	0.3-1.5	12	11	91.67	8	4	50.00	8	5	62.50	6	3	50.00
ISSR13	0.3-1.5	15	11	73.33	10	3	30.00	15	10	66.67	12	5	41.67
ISSR14	0.3-2.0	13	8	61.54	12	4	33.33	10	5	50.00	9	4	44.44
ISSR20	0.3-2.0	15	11	73.33	12	5	41.67	15	8	53.33	12	3	25.00
ISSR23	0.2-1.0	10	7	70.00	7	1	14.29	10	3	30.00	9	4	44.44
ISSR24	0.4-1.0	10	10	100.00	9	5	55.56	8	3	37.50	8	4	50.00
ISSR25	0.2-2.0	13	11	84.62	6	2	33.33	9	4	44.44	10	4	40.00
ISSR28	0.6-1.5	4	0	–	4	0	–	4	0	–	4	0	–
ISSR36	0.3-1.5	10	8	80.00	5	2	40.00	7	4	57.14	7	2	28.57
ISSR38	0.4-1.5	15	13	86.67	9	5	55.56	13	5	38.46	13	5	38.46
ISSR43	0.3-2.0	8	0	–	8	0	–	8	0	–	8	0	–
ISSR44	0.3-1.0	6	0	–	6	0	–	6	0	–	6	0	–
ISSR46	0.4-3.0	15	14	93.33	6	3	50.00	8	5	62.50	8	5	62.50
UBC879	0.6-1.3	6	4	66.67	4	1	25.00	5	1	20.00	4	2	50.00
UBC881	0.3-1.0	10	7	70.00	5	1	20.00	10	6	60.00	13	12	92.31
Sum		162	115	70.99	111	36	32.43	136	59	43.38	129	53	41.09

[a] = Total number of bands.
[b] = Number of polymorphic bands.
[c] = Percentage of polymorphic bands.

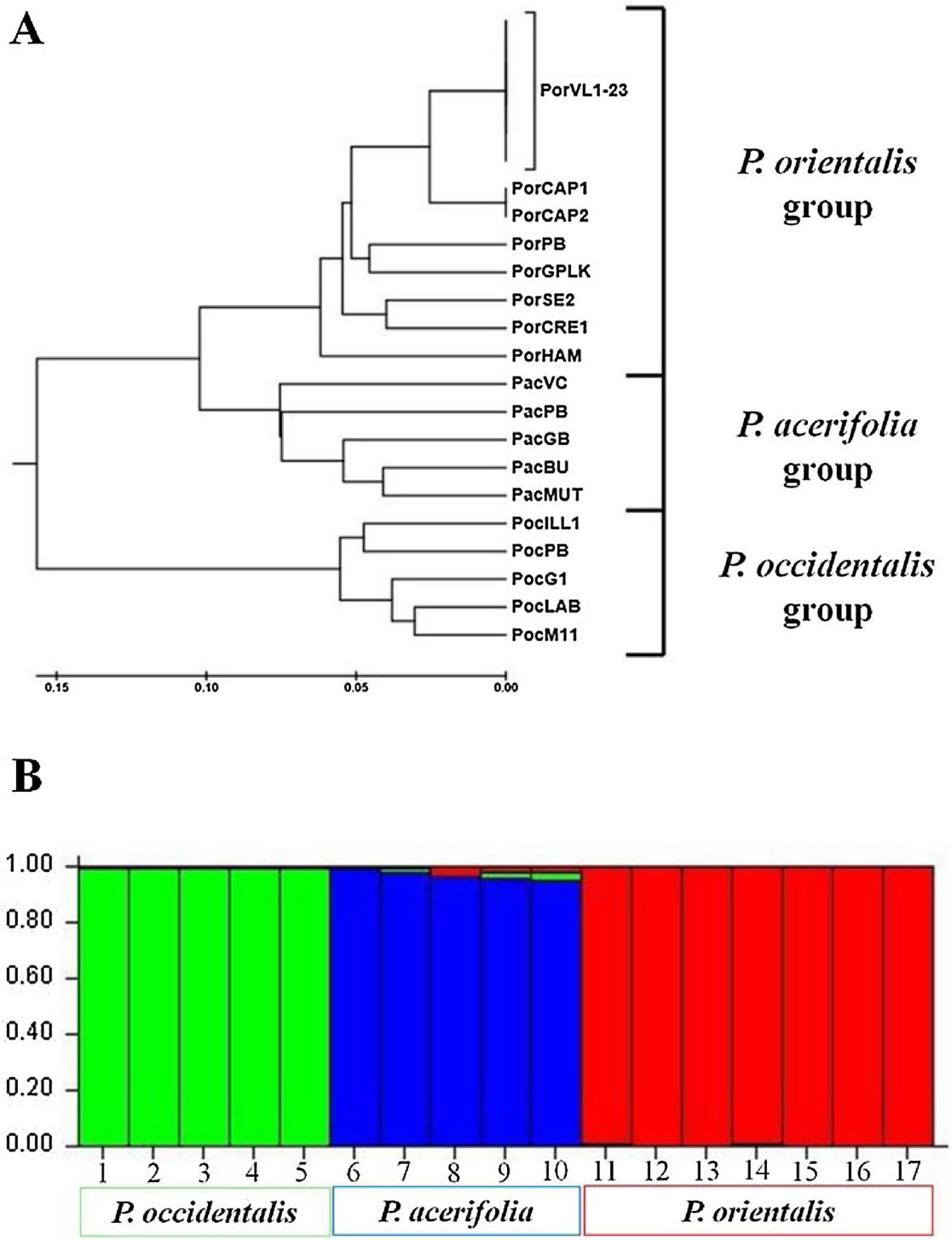

Fig. 5. Clustering of *Platanus* genotypes reported in Table 1, based on 15 ISSR markers. A) UPGMA dendrogram of Nei and Li dissimilarity index between the analyzed *Platanus* genotypes; B) bar plot of Q (estimated membership coefficient for each individual) for the same genotypes (K = 3). Each bar represents a single *Platanus* genotype and colors code the proportion of membership to each plane species: Green = *P. occidentalis*, blue =*P. acerifolia* and red = *P. orientalis*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Vegetative propagations

3.4.1. Woody cuttings

A total of 11 plane trees (PorVL1, 3–9, 14–15, 23 in Fig. 4) were used for woody cutting vegetative propagation. No significant differences in terms of rooting capability were observed between cutting from epicormic shoots 1–2 years old (T-test; P = 0.76). Rooting capacity and the number of roots per cutting of the 11 tested individuals were strictly associated with the phytosanitary status of the mother plant (Fig. 6A). Indeed, the only plant belonging to the 0 phytosanitary class, PorVL7, showed the highest values of rooting (80%) and a significant increase in the root number per cutting (5.25 ± 1.17; Chi square, P = 0.05; Fig. 6B). Moreover, 50% of the fairly symptomatic plants, PorVL 3–5, presented values of a rooting percentage higher than 40% and about 2 roots per cutting (Fig. 6C). These plants, together with PorVL7, are located in the first of the ascending terraces, near the

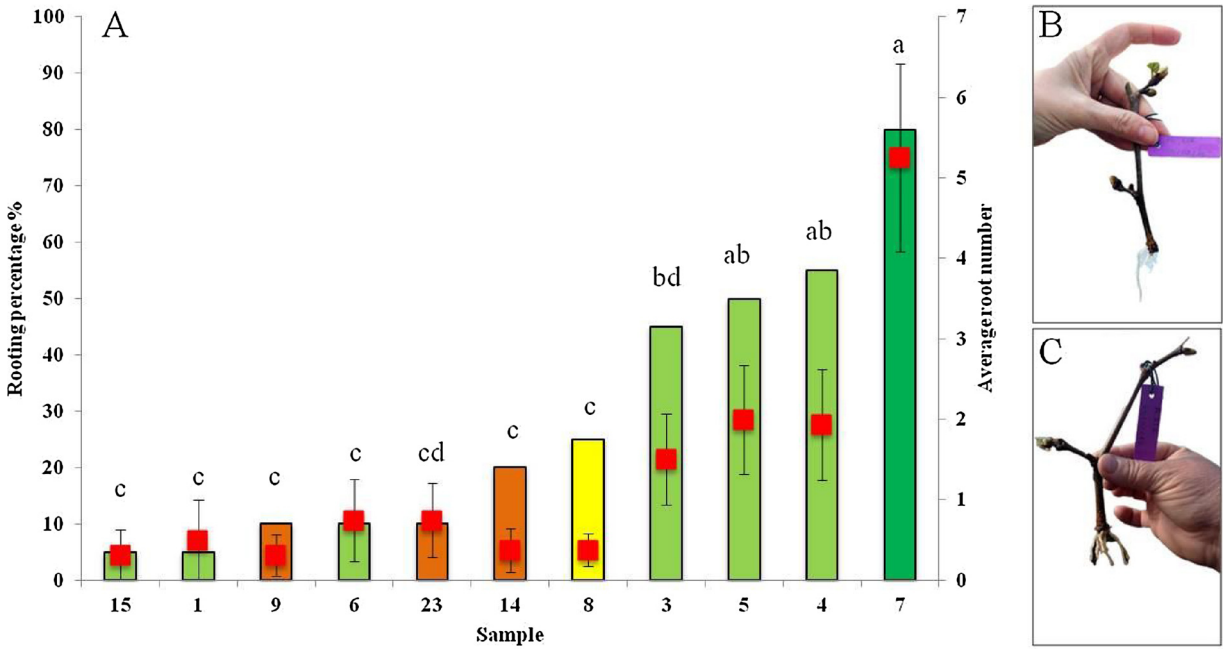

Fig. 6. Rooting percentage (main axis, bars) and average root number per cutting (secondary axis, red squares) of 11 individuals from different phytosanitary classes (0, dark green; 1, light green; 2, yellow; 3, orange) (A); example of rooted woody cutting from healthy (B) and fairly damaged trees (C). Same letters indicate no significant differences in rooting percentage ($P > 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fontana dei Lumini, where probably the water content of the soil is higher than other sectors of the garden. Increasing decay of the plants or different edaphic conditions lead to a very low rate of rooting (< 25%).

3.4.2. Micropropagation

PorVL1, 3–7, 11, 15 (Fig. 4) were the 8 plants used for micropropagation. *In vitro* culture establishment was characterized by high level of initial contamination (> 90%), except for PorVL4-5 and PorVL7 plants (70–80 %; Fig. 7A). The media employed during the multiplication stage produced different results. In fact, within one month microshoots grown on MS and GD appeared stunted and turned respectively vitrescent and reddish, while the material in WPM was healthy even if with a multiplication rate next to zero. Conversely, a good rate of multiplication was obtained with KOP, KP, and KROP media, with KOP showing the highest value ($P = 0.001$). KROP showed higher values than KP for shoots elongation ($P = 0.0009$; Table 5). Nevertheless, within 3–4 months in the multiplication stage, the clumps in KP and KOP media turned yellow and vitrescent (Fig. 7B). For this reason only the clumps in KROP were able to elongate and root (Fig. 7C) after the reduction of BAP and sucrose, combined with the introduction of IBA in the substrates. Finally, 40 rooted plants obtained from KROP were successfully acclimatized (Fig. 7D).

The molecular analyses carried out by using 15 ISSR markers (see Fig. 1 in Kuzminsky et al., 2018) showed that the 10 micropropagated plants studied maintained genetic fidelity respect to the PorVL5 mother plant after 3 year of *in vitro* culture.

4. Discussion

Veteran trees, such as oaks and plane trees, contribute to the unique assets of Italy's cultural heritage of Renaissance Villas. Oriental plane tree (*P. orientalis*) was one of the most prized plants of antiquity. Since ancient times it was commonly planted in public squares for shadow, as reported in Persian sacred books and also in the Bible (de Berenger, 1965). Indeed, for its mythological, religious and philosophical values *P. orientalis* was included among the wonders and antiquities of ancient Greece (de Berenger, 1965). Romans made it the ornament of their pleasure gardens (Daubenton, 1765). In the Italian peninsula the species was reintroduced in the 14th century and it was the only species of the genus *Platanus* employed in European historical park and gardens untill the 17th century (de Berenger, 1965). Afterwards, the introduction of the Northern American *P. occidentalis* occurred in 1636–1637 by the English gardener John Tradescant the Younger (1608–1662), and led to the hybridization between the two species (Henry and Flood, 1919; Gerhold and Frank, 2002). The vigorous hybrid London plane tree (*P. acerifolia*) gradually substituted oriental plane trees due to its wider resistance to abiotic and biotic (mainly *Ceratocystis platani*) stresses. *P. orientalis* characterized the Italian Renaissance garden style; in fact their presence is reported in most of the historical villas such as Villa Lante della Rovere in Viterbo, Villa d'Este in Tivoli, Palazzo Farnese in Caprarola and Villa Aldobrandini in Frascati (Dinelli et al., 1999). Innovative *ad-hoc* solutions should be studied to suggest new and sustainable approaches for their conservation.

To the best of our knowledge, this paper describes for the first time a protocol based on an interdisciplinary approach (COVE) applied to the management and preservation of the green asset of Renaissance villas, combining historical and bioecological data, on an upgradable GIS platform (Fig. 1). The base core of the COVE model can be applied to different veteran trees in varying landscapes, identifying the appropriate methodologies to be used for the species selected as symbol trees.

This study focused on the 23 plane trees currently present within the formal garden of Villa Lante, Viterbo. The asset inventory dated 1603 listed a total of 56 plane trees (Frommel, 2005). However, the original planting design dating 1576 is unknown. The original planted material is attributable to the *P. orientalis* species, since *P. occidentalis* was introduced into Europe later and the first specimens of *P. acerifolia* originated in the 17th century (Henry and Flood, 1919; Besnard et al., 2002). It is important to note that until now no scientific studies were devoted to explore the taxonomy of the plane trees of Villa Lante and their correspondence with the original material planted in its formal garden. The analysis of specific morphological traits suggests that the studied plants belong *to P. orientalis*. The taxonomy of the plane trees of Villa Lante was confirmed also by the amplification of the *LEAFY* gene,

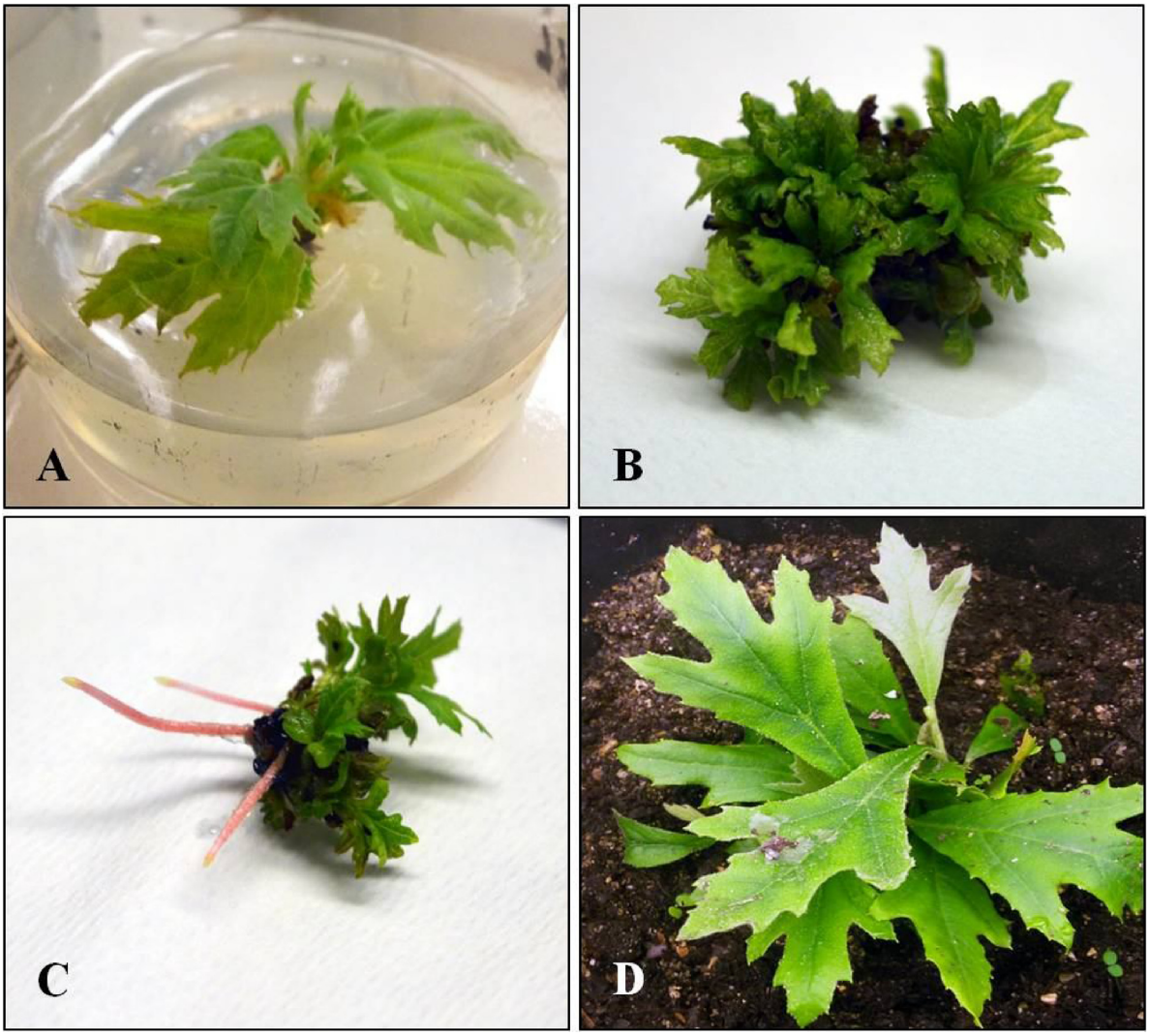

Fig. 7. (A) *In vitro* establishment: healthy shoots coming from bud disclosure in KP; (B) Multiplication phase: vitrified micro-shoots in KP; (C) Rooting phase: shoot clump in KROP; (D) Acclimatized material in *post-vitro*.

Table 5
Average number of new axillary shoots/explant (N = 5) per explant, and average length of the three highest shoots per explants (N = 15) after 1 month in the culture medium. Data are presented as means ± SE.

	KP	KOP	KROP
Axillary shoots	7.80 ± 0.58b	9.80 ± 0.37a	3.60 ± 0.40c
Shoot length	1.21 ± 0.05b	1.29 ± 0.05ab	1.54 ± 0.07a

a single copy nuclear gene in diploid angiosperms (Frohlich and Meyerowitz, 1997). Sequences of different regions of the *LEAFY* gene were used to study the phylogenetic relationships within the *Platanus* genus (Feng et al., 2005) and to confirm the hybrid origin *P. acerifolia* specimens by detecting specific *P. orientalis* and *P. occidentalis* alleles (Pilotti et al., 2009). All the 23 plane trees analyzed showed the presence of a DNA fragment peculiar of *P. orientalis* (about 700 bp) (Fig. 2), sharing high sequence identities (> 97%) with the *P. orientalis LEAFY* sequences in the NCBI database. Moreover, the amplification of the *LEAFY* gene allowed us to differentiate most of the accessions belonging to the *P. occidentalis*, *P. orientalis* and *P. acerifolia* species used as references, with the exception of the *P. acerifolia* accessions PacMUT and PacBGB, in which only the *P. orientalis* DNA fragment was observed (Fig. 3), confirming data from literature (Pilotti et al., 2009). These results could be explained by more frequent backcrosses of *P. acerifolia* with *P. orientalis* than with *P. occidentalis* in Europe, probably because of the disappearance of *P. occidentalis* in the early nineteenth century (Besnard et al., 2002).

The ISSR primers used in this study allowed us to distinguish without ambiguity the species *P. acerifolia, P. orientalis* and *P. occidentalis* (Fig. 5) and revealed significant variations among accessions of the three species (Table 4). However, *P. occidentalis* displayed a lower level of polymorphism than *P. orientalis* and *P. acerifolia* (Table 4), confirming previous data based on molecular analyses (Besnard et al., 2002). Moreover, pairwise estimates of similarity (Table S4) and the UPGMA dendrogram obtained on the basis of ISSR markers (Fig. 5A) indicated a closer genetic relationship between *P. acerifolia* and *P. orientalis* than between the hybrid species and *P. occidentalis*. These results confirm a more important contribution of *P. orientalis* than *P. occidentalis* in the constitution of the hybrid species *P. acerifolia.* As discussed previously, this phenomenon could be due to the high potential for backcross with *P. orientalis* (Besnard et al., 2002). Finally, ISSR analysis showed a substantial genetic homogeneity of all the plane trees from Villa Lante (Fig. S1) and confirmed their membership to the species *P. orientalis* (Fig. 5). This finding, combined with the late maturity stage of the plants, suggests that all specimens present today could be the original plants planted by Cardinal De Gambara in the 16th century. The dating of plane trees with current non-destructive

methodologies, such as increment borer, is difficult to be utilized due to the likely presence of stem decays, but it is well know that *P. orientalis* can survive more than 500 years (Vigoroux, 2007). Probably, plants used for the establishment of the formal garden have been obtained from seeds harvested from genetically homogeneous individuals as present in Southern Italy (Rinaldi, 2016) or by vegetative propagation from a single mother plant.

The capacity for vegetative propagation in trees varies greatly between species and genotypes, and it is affected by both environment and physiological state. Multi-century trees are particularly difficult to propagate for lack of juvenile tissues. In mature trees epicormic shoots, which can sprout along the stem, particularly in pruned trees, could be a suitable source of juvenile material (Ferrini, 2006). The species belonging to the genus *Platanus* produce this type of branches, which were successfully employed to micropropagate *P. acerifolia* (Alegre et al., 2015). In our study, epicormic shoots were collected in order to set up two vegetative propagation systems associated with a low risk of mutation: woody cuttings and micropropagation by organogenesis (node culture).

Woody cutting is considered the best method to clone oriental plane trees starting from young individuals (Grolli et al., 2005), however there is a lack of knowledge of explants from multi-century trees. Our data confirms the high efficacy of the protocol and showed that the contrasting rooting capability mainly depends on the phytosanitary status of the mother plant and edaphic conditions. In fact, no genetic variability of the 11 specimens tested in this study was detected by molecular analyses. When tissues are collected from apparently healthy plant, rooting percentage can reach high values (80% of rooting for the specimen PorVL7) confirming data from the literature (Vlachov, 1988; Fig. 6A). A reduction of transplanting stress could be achieved by using a jiffy pot inside the bench with a heat bottom device according to Arène et al. (2002).

Concerning the micropropagation of *Platanus* spp., our findings are in agreement with the literature. Several authors, showed a difficulty in the establishment of the culture of *P. acerifolia* due both to the contamination by latent endophytes to a high content of ammonium nitrate (Gjuleva and Atanasov, 1994; Grolli et al., 2004; Alegre et al., 2015). Indeed, in the present study the rich formulation of MS and KP media (Table 2) in terms of macronutrients combined with the highest level of NH_4^+ (20.61 mM) leads to vitrification within 1 or 3 months, respectively. Conversely, the absence of ammonium nitrate and a high content of calcium nitrate in KOP substrate resulted in a significant higher multiplication rate with respect to KP and KROP (Table 5), but the microshoots turned yellow within 3–4 months. Therefore *P. orientalis* needs a certain content of NH_4^+ for prolonged culture as ascertained by the green and healthy status of the microshoots obtained in WPM and KROP (ammonium content, 5.00 mM). Unfortunately, the extremely low multiplication rate of the microshoots in these two media, characterized by a quite similar mineral composition, is difficult to explain although in agreement with Arène et al. (2002), who employed WPM in the only paper devoted to *P. orientalis* micropropagation. The stunted growth of *P. orientalis* in GD medium, successfully used by Gjuleva and Atanasov (1994) for *P. acerifolia,* could be due to a higher need of *P. orientalis* for magnesium, calcium, and sulfur, which would also explain the superior performance of KROP respect to KP in the shoot elongation (Table 5). In conclusion, micropropagation of *P. orientalis* needs the following steps: i) one month in KP for the establishment of the culture; ii) the employment for another month of KOP medium added with BAP (0.50 mg L^{-1}) to induce an elevated number of axillary shoots; iii) 2–3 months in KROP medium added with BAP (0.050 mg L^{-1}) and IBA (0.020 mg L^{-1}) in order to favor shoot elongation and rooting. Furthermore, our data showed that the micropropagated plants of *P. orientalis* were genetically stable after 3 years. However, organogenic differentiation is reported to be prone to somatic variation (Pontaroli and Camadro, 2005), even if with low risk as demonstrated for *P. acerifolia* after 8 years (Huang et al., 2009).

The multiplication of veteran trees *in vitro* conditions offer the possibility grow plants in large numbers in a short period and free of pest and pathogens such *C. platani* and Ambrosia beetle, often associated with nursery stocks (Ocasio-Morales et al., 2007; Tubby and Pérez-Sierra, 2015) Results from the survey undertaken in the present study indicate that oriental plane trees examined were not infected by *C. platani*, even if susceptible. Interestingly, CSD of *Platanus* spp. is a widespread disease in Italy, observed also in an urban park in Viterbo. Since the first confirmation of the disease in 1972 (Panconesi, 1972), the pathogen had already destroyed the tree rows of several monumental avenues, including the boulevard which connected the Reggia di Caserta with Naples (Cristinzio et al., 1973). Plants for planting, wood, machinery (including pruning/cutting tools), root anastomosis and natural spread through wind are considered as mechanisms for the spread of the pathogen into an area (Jeger et al., 2016). The risk of the introduction of the pathogen in historical villas is very high if the management of the garden would include tree replacement and use of possibly contaminated machinery. Thus, proper management plans should include on the planting of young plane trees, and using material from the local germplasm, certified for the absence of pests and pathogens, before the original individuals disappear. These conditions are easily guaranteed if *in vitro* material is used. Moreover, micropropagated material can be treated with PGPR (Plant Growth Promoting Rhizobacteria), which are beneficial microorganisms for the obtainment of more vigorous plants against abiotic and biotic stress (Vettori et al., 2010; Bhattacharyya et al., 2016).

5. Conclusions

The interdisciplinary analytical framework, COVE, here applied to the monumental garden of Villa Lante, provided information and specific methodologies useful for a sustainable strategy for the conservation and management of veteran plane trees. The model can be adapted to other scenarios, recognizing that each garden is unique and contextually situated. In particular, through a GIS tree inventory it is possible to: i) quantify the tree asset and identify risky trees in order to plan management practices (*e.g.* pruning, removal, chemical treatments, *etc.*); ii) avoid risks for people, buildings and artifacts and preserve the germplasm; iii) prevent the unnecessary removal or disfigurement of amenity trees, preserving the aesthetics of the garden; iv) conserve the habitats provided by the old trees as long as possible; v) verify the susceptibility to pests even if not present in the area, including quarantine pathogens, of veteran trees; vi) identify the best trees to be propagated for the germplasm conservation and production of material useful for replacement; vii) allow the managers of monumental complexes to make more effective use of available funds.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ufug.2018.07.022.

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