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Electronic identification systems for reducing diagnostic workloads after disease outbreak

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Diagnostic tests for grapevine viruses subjected to phytosanitary rules involve a heavy workload for plant protection services and laboratories. Propagation schemes enable nurseries, where mother plants (MPs) are cultivated, to be linked to batches of certified plants (CPs). This approach entails post-production checks of MPs once infection occurs in CPs. However, this traceability system is not tight and follow ups are demanding. This study assessed radio frequency identification (RFID) tagging of plants in terms of its ability to reduce laboratory workloads for nursery health checks. RFID-tagged plants (RFID-CPs) were produced from individually tagged MPs (RFID-MPs) or row-tagged MPs (RFID-ROW, a less expensive approach). In a 10-year case study, the health status of CPs and RFID-CPs were assessed and the occurrence of infections then led to health checks in MPs, RFID-MPs or RFID-ROWs. Laboratory workloads were evaluated by considering two sampling methods (single or pool sampling). Using single sampling, the workload was reduced by 93–98% in RFID-ROW or RFID-MP checks compared to the conventional approach. Considerable reductions in workload due to the tagging system (93–96%) were also observed using pool sampling. Traceability of CPs and MPs using RFID reduces laboratory workloads, and supports emergency measures that can be taken to stop any unsafe sales of plants after a virus outbreak.

Keywords: grapevine, MolU, radio frequency identification, virus

Introduction

Field data collection, processing and analysis can be carried out by using farm management information systems (FMISs), thus assisting growers in managing farms and orchards (Fountas *et al.*, 2015). Cloud-based FMISs may represent an efficient marketplace for services for stakeholders (Kaloxylos *et al.*, 2014), given that they improve operational planning and optimize the workload (Fountas *et al.*, 2006; Sørensen *et al.*, 2011; Ampatzidis *et al.*, 2014).

This approach works well with the digitalization of plant data, which can then be used for health monitoring, sample collecting and retrieving health information (Vai, 2005; Kumagai & Miller, 2006; Thrane, 2008; Cunha *et al.*, 2010), as well as identifying and verifying agrochemicals in food traceability systems (Peets *et al.*, 2009). In certified plant propagation and breeding programmes, electronic identification technology can be a useful tool for managing risks related to environmental impact of production systems, chemical residues, and the spread of plant pathogens, encouraging a shift toward a radio frequency identification device (RFID) system (Luvisi *et al.*, 2012a).

The loss, removal or damage of traditional plant labels at any stage of production can result in a mother plant that has no known history. This is particularly problematic because of the many viruses, viroids, phytoplasmas and other systemic pathogens that can infect propagative material. To limit the risk of pathogen spread, diagnostic protocols for grapevine viruses subjected to phytosanitary rules are used. However, these protocols significantly add to the workload of laboratories of plant protection services (Faggioli *et al.*, 2013).

Using electronic devices that link propagative materials to mother plants (individually or row-related) could support emergency measures that can be taken to stop the unsafe sale of plants by nurseries with infected mother plants. Basic material and derived certified material from the grapevine production line can be safely tagged with RFIDs to establish mother plant vineyards.

In a previous paper, Luvisi *et al.* (2012b) found no detrimental performance in terms of vegetative growth and bud production for mother plant vineyards from the first phase of production life, and tags were readable to check identities after 4 years from implanting. However, tags need to be reliable in the long term so that the grapevine production line can be traced.

This paper reports a 10-year case study. The health status of certified plants was assessed and, once virus

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infection occurred, the related mother plants were analysed in a post-production health check. Health monitoring at nurseries should establish whether infections of certified plants occurred after transplanting or were due to the use of infected propagative materials, with the potential unsafe sale of plants by nurseries.

The laboratory workload for virus assays in RFID-tagged vineyards and nurseries was estimated and compared to a conventional production line. The reliability of tags 10 years after grapevine tagging was also evaluated (Luvisi *et al.*, 2010) using various readers.

Materials and methods

RFID tagged vineyards

Grapevine tagging of mother plants (MPs) for nurseries was carried out following Procedure A, as described in a previous paper by Luvisi *et al.* (2010). This method consists of microchip insertion after direct drilling of the pith from the distal cut of the rootstock just before grafting, followed by microchip localization below the grafting point (Fig. 1). Trials involved grafted cuttings of *Vitis vinifera* 'Sangiovese' (clone I-SS-F9-A5-48), 'Colorino' (I-US-FI-PI-10) and 'Vernaccia di San Gimignano' (I-V-P-6) grafted in 2007 on 1103 Paulsen rootstock (*Vitis berlandieri* × *Vitis rupestris*), supplied by the Associazione Toscana Costitutori Viticoli (TOS.CO.VIT.). Transponder glass tags were used, 2.1 mm diameter and 12 mm long, working at a frequency of 125 kHz (InterMedia Sas). Tagged MPs (RFID-MP) were transplanted in 25-plant rows (one row per cultivar). Each row was also identified by an RFID tag (RFID-ROW).

Grapevine tagging of certified plants (CPs) for vineyards was carried out in the same ways as RFID-MPs, but shorter glass tags were used (10 mm long; EM Microelectronic). Cuttings from previously described RFID-MPs were used in 2012 and grafted onto Kober 5BB rootstocks (clone I-AGRI/20; TOS.-CO.VIT.). Starting from three RFID-MPs, a total of 120 CPs per cultivar were produced (1:40, MP:CP), linking propagative data of each group of 40 RFID-CPs to the related RFID-MPs (Fig. 2a). RFID-CPs were also linked to RFID-ROW, to evaluate a less expensive method for tracing MPs (Fig. 2c). Rootstock cuttings were obtained from a 30-plant untagged vineyard (Fig. 2b), due to the lack of available tagging procedures for rootstocks. Grapevines were transplanted in the vineyard in 2013.

Untagged CPs were used as a control and transplanted in the same vineyard as the tagged CPs. They were derived from untagged MPs belonging to three nursery vineyards of various sizes (1200, 115 and 75 MPs of Sangiovese, Colorino and Vernaccia di San Gimignano, respectively; Fig. 2d,e). Rootstock cuttings were obtained from a vineyard with 30 untagged MPs.

Tags were electronically read every 2 years by means of a 14 digit identification number using a compact flash reader (CFR) connected by an SD slot to a palm-PC (Dell Axim X51) or using a wand reader (WR; LiveTrack, Syscan RS Inc.). Data recovery (tag ID number) from CFR was performed using a palm-PC containing a database specifically programmed using SprintDB Pro (KaioneSoft), while stock software was used for retrieving data with WR. Designing a specific software application to associate IDs from RFID-CP to RFID-MP or RFID-ROW was beyond the scope of this trial, thus it was carried out manually using an open access datasheet. Tag reliability was expressed as readable plants out of total tagged plants.

Plant health monitoring

Grapevine samples were collected in January 2016 from CPs (tagged and untagged) and tested for the presence and distribution of viruses covered by EU regulations (directive 2005/43/EC) and Italian regulations (decree DM 07/07/2006). With regard to Italian regulations on grapevine health assays, in addition to single samples, pools of up to five homogeneous samples (one per plant) may be tested for viruses. Both sampling methods (single sampling or pool sampling) were considered for tagged or untagged cultivar MPs.

Following health results on tagged or untagged CPs, virus assessment was carried out on tagged or untagged MPs, respectively. As mentioned above, untagged CPs came from untagged MPs belonging to three nursery vineyards of various sizes, from 75 to 1200 MPs depending on the cultivar. Thus, individual infections of untagged CPs led to 75–1200 single samplings (or 15–240 pool samplings of MPs) that needed to be tested for viruses (Fig. 2d,e). A similar approach was carried out when checking rootstocks (Fig. 2b). On the other hand, a single infected tagged CP linked the health check to an individual MP (Fig. 2a) or to an RFID-tagged row of 25 MPs (25 individual

Figure 1 (a) Grapevine tagging with RFID tags (Luvisi *et al.*, 2010). (b) Location of tags (in rectangle) in 8-year-old grapevine mother plants. (c) Reading of certified plants with RFID tags (Luvisi, 2016).

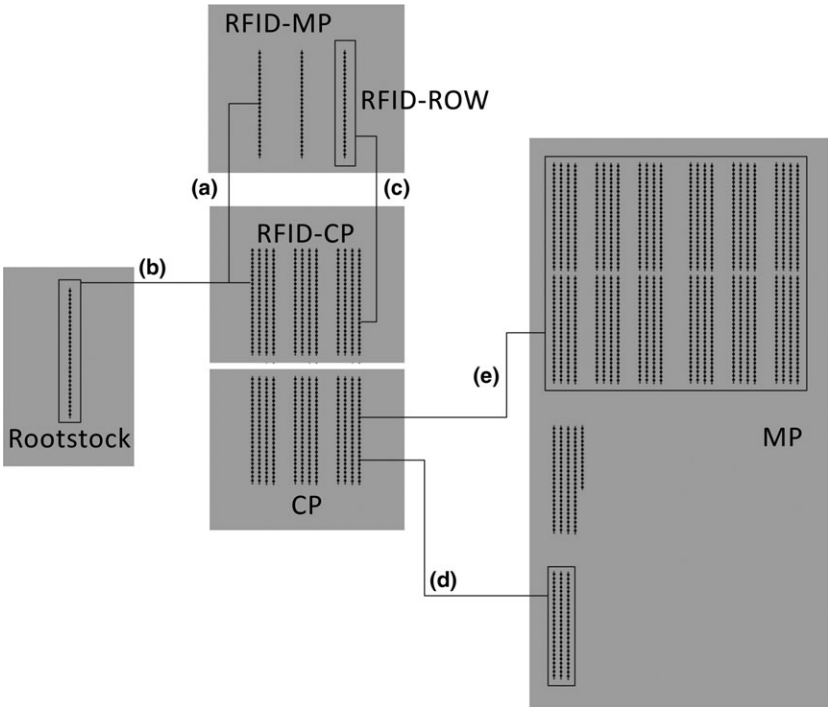

Figure 2 Relationships between RFID-tagged certified plants (RFID-CP) or untagged plants (CP) in vineyards, and RFID-tagged mother plants (RFID-MP) or untagged plants (MP). (a) RFID-CPs individually linked to individual RFID-MPs and (b) to untagged rootstock rows of 30 plants. (c) RFID-CPs individually linked to RFID-tagged rows (RFID-ROW) of 25 MP. (d) Untagged CPs linked to small MP vineyards (75 plants) or (e) larger vineyards (1200 plants).

samplings or five pool sampling of MPs; Fig. 2c), potentially reducing the laboratory tests compared to those needed to check untagged plants.

Virus analysis and laboratory workload

To account for the possible uneven distribution of viruses within a vine, samples from at least four different shoots were randomly collected and combined. Total RNA was extracted from cambial scrapings of lignified cuttings (2 g) using RNeasy Plant Mini kit (QIAGEN) protocol, modified according to MacKenzie *et al.* (1997). The extracted RNA was then reverse-transcribed into cDNA using the iScript cDNA Synthesis kit (Bio-Rad) and primers and probes for *Grapevine leafroll associated-virus 1* (GLRaV-1) and 3 GLRaV-3 (Osman *et al.*, 2007), *Grapevine fanleaf nepovirus* (GFLV), *Grapevine fleck virus* (GFkV) (Osman *et al.*, 2008), *Grapevine virus A* (GVA; Osman & Rowhani, 2008) and *Arabis mosaic virus* (ArMV; Wei *et al.*, 2012) detection were used. For each sample, 2 μL of cDNA were amplified in a total volume of 20 μL containing 1× SsoFasto probe Master Mix (Bio-Rad) and 0.4 μM of each primer and probe. Reactions were performed in a CFX96 Real-Time thermocycler (Bio-Rad). Data were analysed by measuring the threshold cycles (C_t), and analysed using SigmaPlot v. 11 (Systat Software).

In order to evaluate laboratory workloads for (i) infected RFID-CPs with direct links to individual RFID-MPs (Fig. 2a); (ii) infected RFID-CPs with links to RFID-ROW (Fig. 2c); and (iii) infected untagged CPs linked to whole MP vineyards (Fig. 2d,e), the molecular unit (MolU) method was applied (Stenhouse, 2011). The method is applied by the UK Genetic Testing Network and uses an 'amplicon' or equivalent as the basic unit. In accordance with this method, RNA extraction and cDNA synthesis were weighted 1 MolU (no differences in sample preparation/RNA extraction between single or pool samples were found). Similarly, single virus detection was weighted 1 MolU.

The total number of virus tests carried out on MPs may vary depending on the number of viruses retrieved in CPs and in relation to the sampling method (single or pool sampling) of MPs.

Statistical analysis

The effects of treatments and successful read rate were compared by analysis of variance in a random design using CoStat v. 6.203 (CoHort Software). Duncan's multiple range test at 5% level was calculated for comparison. Data as percentages were normalized by arcsine square root transformation.

Results

Plant health monitoring

With regard to MPs tagged with longer tags, the less powerful reader (CFR) was able to read 80% of tagged plants that had been tagged at transplantation 6 years ago (Fig. 3). At this stage, the mean trunk diameter was above 35 mm. After 8 years, 80% of tags were still readable. The more powerful reader (WR) managed to read plant IDs even after 8 years, when mean trunk diameter was about 40 mm. With regard to CP plants that were labelled with tags with shorter antennae, tag reliability had decreased after transplanting, although reliability was higher than 90% after 2 years using WR (Fig. 3).

Health monitoring of CPs led to similar disease rates in both RFID-tagged and untagged vineyards, with two viruses (GVA and GLRaV-3) found out of six checked. GVA was found in 1.6% and 2.0% of RFID-CPs and CPs, respectively. GLRaV-3 was also found in both CP vineyards, achieving an infection rate of 2.1% in RFID-

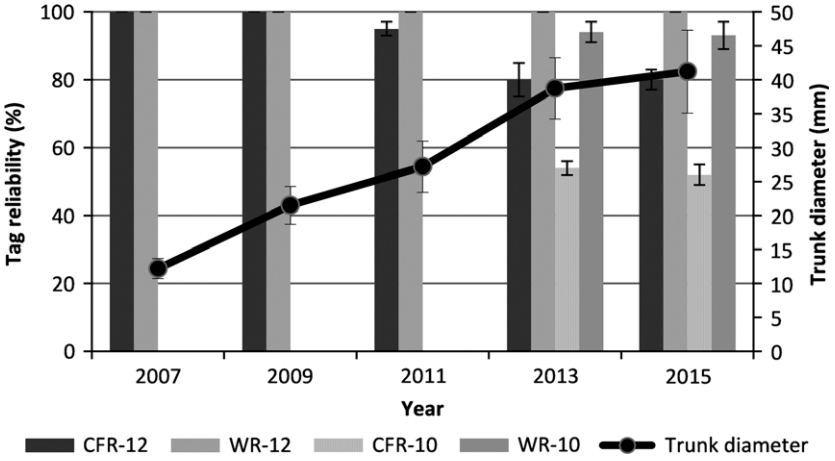

Figure 3 Tag reliability (readable plants, out of total tagged plants, %) observed every 2 years from transplanting. Plants transplanted in 2007 were tagged with 12-mm long tags, and read with a compact flash reader (CFR-12) or a wand reader (WR-12). Plants transplanted in 2013 were tagged with 10-mm long tags, and read with a compact flash reader (CFR-10) or a wand reader (WR-10). Mean trunk diameter (mm) was measured in 2007–15.

CPs and 1.6% in CPs. Differences in virus incidence were observed among cultivars, but infection rates were similar in both vineyards. Details of infection among cultivars are reported in Tables 1 (CP) and 2 (RFID-CP).

Laboratory workload

With regard to CPs derived from untagged plants, the number of MPs to check depended on the size of the MP vineyards and varied among cultivars (Table 1). With regard to RFID-CPs derived from tagged rows, the number of MPs to check did not depend on the number of infected CPs; instead 25 MPs were checked for each cultivar, which constituted one RFID-ROW (Table 2). With regard to RFID-CPs individually linked to RFID-MPs, the infection of one or more plants within a cultivar subgroup led to the identification of one RFID-MP. Thus, the number of MPs to check depended on the health status of the derived CP, but could never exceed three RFID-MPs per cultivar (Table 3).

Both traceability systems and sampling methods had a heavy impact on the laboratory workload (Tables 1, 2 & 3). Decrements in samples to be managed and the number of tests were calculated if RFID-CPs were linked to an RFID-ROW (Table 2) compared to untagged plants (Table 1). Using the single sampling method, the laboratory workload was reduced by 93%. A stronger effect was observed in RFID-CPs individually linked to RFID-MPs, with 102 MolU compared to 4285 MolU for monitoring untagged MPs, leading to a 98% reduction in the workload (Table 3).

Pool sampling saved time. In fact, when applied to untagged vineyards, it decreased the MolU by 80%. The effect of sampling method was the same for RFID-ROW, while it had less impact for RFID-MPs (a 70% reduction in MolU). Tagging also led to a considerable reduction in laboratory workload when pool sampling was adopted. The RFID-ROW method caused a 93% reduction in MolU compared to untagged plants, and there was a 96% reduction when RFID-MPs were used.

Time and costs for sampling should also be considered. Although standardized methods to evaluate sampling workload were not assessed, the health checks for untagged CP entailed collecting 1420 MPs and 284 samples (Table 1). On the other hand, with the tagging method, the health status of only five RFID-MPs needed to be investigated in order to establish either a post-transplanting infection or unsafe production of CPs (Table 3). Even if the health status of RFID-CP had been worse than reported in this paper, no more than nine RFID-MPs would have been affected by post-production assay.

Table 1 Size of groups of certified untagged plants (CP) and relative untagged mother plants (MP).

					Single plant sampling[b]			Pool sampling[b]		
Clone	CP	MP	GVA[a]	GLRaV-3[a]	Samples	Tests	MolU	Samples	Tests	MolU
I-SS-F9-A5-48	76	1200	2	3	1200	2400	3600	240	480	720
I-US-FI-PI-10	83	115	2	1	115	230	445	23	46	69
I-V-P-6	88	75	1	0	75	75	150	15	30	45
I-AGRI/20[c]	—	30	—	—	30	60	90	6	12	18
Total							4285			852

[a]Numbers of CPs positive for viruses (GVA or GLRaV-3) are reported.
[b]Numbers of samples (obtained by single samplings or pool samplings), virus tests and molecular units (MolU) were calculated considering potentially infected MPs.
[c]Rootstock.

Table 2 Size of groups of certified RFID-plants (CP) and mother plants (MP) associated with RFID-tagged rows.

					Single plant sampling[b]			Pool sampling[b]		
Clone	CP	MP	GVA[a]	GLRaV-3[a]	Samples	Tests	MolU	Samples	Tests	MolU
I-SS-F9-A5-48	61	25	1	3	25	50	75	5	10	15
I-US-FI-PI-10	99	25	2	2	25	50	75	5	10	15
I-V-P-6	83	25	1	0	25	25	75	5	10	15
I-AGRI/20[c]	—	30	—	—	30	60	90	6	12	18
Total							315			63

[a]Numbers of CPs positive for viruses (GVA or GLRaV-3) are reported.
[b]Numbers of samples (obtained by single samplings or pool samplings), virus tests and molecular units (MolU) were calculated considering potentially infected MPs.
[c]Rootstock.

Table 3 Size of groups of certified RFID-plants (CP) and relative RFID-mother plants (MP).

					Single plant sampling[b]			Pool sampling[b]		
Clone	CP	MP	GVA[a]	GLRaV-3[a]	Samples	Tests	MolU	Samples	Tests	MolU
I-SS-F9-A5-48	12	1	1	2	1	2	3	1	2	3
I-SS-F9-A5-48	21	1	0	1	1	1	2	1	1	2
I-SS-F9-A5-48	28	1	0	0	0	0	0	0	0	0
I-US-FI-PI-10	32	1	2	1	1	2	3	1	2	3
I-US-FI-PI-10	31	1	0	1	1	1	2	1	1	2
I-US-FI-PI-10	36	1	0	0	0	0	0	0	0	0
I-V-P-6	27	1	0	0	0	0	0	0	0	0
I-V-P-6	25	1	1	0	1	1	2	1	1	2
I-V-P-6	31	1	0	0	0	0	0	0	0	0
I-AGRI/20[c]	—	30	—	—	30	60	90	6	12	18
Total							102			30

[a]Numbers of CPs positive for viruses (GVA or GLRaV-3) are reported.
[b]Numbers of samples (obtained by single samplings or pool samplings), virus tests and molecular units (MolU) were calculated considering potentially infected MPs.
[c]Rootstock.

Data analysis of untagged CPs was strongly dependent on the size of the related MP vineyards. In this study, the impact of data relative to Sangiovese cultivar (I-SS-F9-A5-48) was significant, even though its size was not abnormal for a grape nursery. However, by limiting the calculation to the cultivars with the lowest numbers of MPs (I-V-P-6) and affected by just one virus, the impact of RFID tagging was also great.

With regard to the single sampling methods, laboratory workload was decreased by 31% and 62% using RFID-ROW and RFID-MPs, respectively. Applying pool sampling methods, the decrease in MolU was 47% and 68% using RFID-ROW and RFID-MPs, respectively. On the other hand, if the number of CPs were higher than in this study, the number of rows (or plants in each row) to be considered would also be higher. In any case, these speculations are likely to affect the untagged plants too, thus impacting management data.

Discussion

Due to widespread viruses (Rizzo *et al.*, 2012, 2015) and phytoplasmas (Marchi *et al.*, 2015), periodical surveys are essential in order to monitor diseases in vineyards in Tuscany (Italy). Although consumers may have little notion about what traceability is and may not be interested in the technical aspects associated with traceability (Giraud & Amblard, 2003), scientific knowledge of the incidence of diseases may lead to the development of tools that can promote trust between farmers and nursery growers (Luvisi, 2016).

Health monitoring strategies require reliable tagging systems. However, low-frequency glass tags have limitations, particularly with regard to reading distance. This issue can be overcome by using more powerful scanners (Bowman, 2005, 2010) or tags operating at higher frequencies, such as UHF tags (Luvisi *et al.*, 2014). The findings here suggest that wand readers (originally designed for livestock) can ensure lifetime reading in grapevine. However, tags with longer antennas need to be used, otherwise reading may only be efficient in the 2 years after transplanting.

The system used for supporting disease monitoring could be integrated with other tools for sampling support (Luvisi *et al.*, 2012c) or with harvest management information systems that provide real-time access to harvest

data (Ampatzidis *et al.*, 2013, 2016). RFID systems can also be part of a wireless sensor network for monitoring agricultural fields (Anisi *et al.*, 2015).

Workload was found to be strongly dependent on the sampling method (individual plants or pools). However, this workload can be dramatically decreased when plants are labelled, individually or by row. Data analysis of untagged CPs was strongly dependent on the size of the MP vineyards – two very different sizes were studied here. In Italian nurseries, monoclonal MP vineyards with hundreds of plants are common for cultivars such as Sangiovese, while the small size of vineyards that cultivate Vernaccia di San Gimignano (less than 100 plants) is characteristic of local varieties.

With regard to the management of tagged plants, the cost of tags (about $1) may significantly impact on plant cost. However, the cost of untagged certified plants may vary greatly from year to year due to market fluctuations. The effect of tagging on the final price (and sale opportunities) requires economic analyses that until now have not been undertaken for plant traceability (with RFIDs or similar devices; Luvisi, 2016). Furthermore, external factors due to control of pest spread should be carefully considered in the economic analysis, as observed in recent pest outbreaks in Europe (Luvisi *et al.*, 2017).

In addition to the cost of tagging, the management of links between individual MPs and CPs, generally for sets of 10 plants, is time consuming, due to the need to stop MP pruning after each plant and distinguish between cuttings from different MPs. Thus, even if the workload in health assessment is definitely low compared to untagged plants, a nursery would have to take into account a significant increase in workload in CP production. Unfortunately, there is no data on the costs of training operators to place RFIDs on plants, nor is there any data on the loss rate of microchips and plants during implanting (Luvisi *et al.*, 2012a). Moreover, the complexity of procedures in identifying plant materials may lead to errors that could invalidate the potential benefits.

The management of cuttings harvested from tagged rows may interfere slightly with common nursery practices, because plant material has to be differentiated between rows, a practice already carried out in most nurseries. This approach can minimize the increase of workload in CP production, and also support traceability and strongly reduce laboratory workloads.

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