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Transformation of kiwi and olive with tobacco osmotin gene confers drought-stress tolerance and provides a health-promoting protein source

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Abstract

Based on climate change projections, salinity and drought are viewed as the most important environmental stresses, the main causes in the decrease of crop production and a major challenge for agriculture. Research efforts should be focused on the selection of more resilient crop genotypes for future agriculture, as traits, such as tolerance to drought, salinity and frost, are lacking in the common commercial cultivars. Transgenic approaches have generated opportunities for crop improvement. Both, "Hayward" kiwifruit vines and "Canino" olive trees, have been transformed with the tobacco osmotin gene under 35S promoter control and tested *in vitro* and in vivo for 10 years. The osmotin gene, which codes for a PR-5 protein, is commonly present in the genome of all plant species, but its expression and protein accumulation are elicited in response to both abiotic and biotic stresses, resulting in effective control of fungal diseases. Interestingly, the osmotin plant protein from tobacco was found to be a homologue of the key human hormone adiponectin, and numerous reports have shown that it is capable of activating the human Adipor1/Sirt1 receptors, thus inducing biogenesis in human cells *in vitro* and providing a new strategy for the treatment of various diseases (diabetes, cancer, central nervous system disorders). Physiological responses to drought stress imposed by PEG8000 (1, 2 and 4 %) were compared *in vitro* in transgenic and control shoots. PEG resulted in oxidative injury, as expressed in increased lipid peroxidation, and significant changes of enzyme activities involved in antioxidative mechanisms. The kiwi and olive transgenic shoots showed better adaptation to drought stress, confirmed by field observation on plants. In addition, the transgenic olive also resulted more tolerant to frost *in vitro* tests. Both transgenic species resulted more tolerant to biotic stress in artificial inoculation tests with *Botrytis cinerea* and *Cadophora luteo-olivacea* on ripe fruit epidermis. The transgenic fruits resulted significantly less rotten as compared to control fruits. Transgenic olive potted plants grown alongside control plants in the same environment showed lower susceptibility to natural infection of *Spilocaea oleagina*, as evidentiated by fewer and smaller spots on the leaves. The creation of improved plants suitable for adverse abiotic and biotic conditions and the opportunity to also use their leaves and fruits as a source of the health-promoting osmotin protein provide amazing perspectives for future research in human health and agricultural biotechnology.

***Keywords*:** osmotin, adiponectin, *SIRT1*, *Actinidia deliciosa* A. Chev, *Olea europaea* L., health, abiotic stress.

1.Introduction

Olive trees and kiwifruit vines are two important fruit crop species, which represent a significant industrial interest for countries in the Mediterranean basin and around the world. Since about 1/4th of the cultivated land in the world is susceptible to damage from salts (particularly sodium

chloride, sulfates of Ca, Na and Mg, potassium chloride, and sodium carbonate) plants resistant to biotic and abiotic stresses are required to meet today's needs. It is known that osmoregulators such as NHX1, which is a sodium antiporter (Zhang and Blumwald, 2001); trehalose; proline, a plant osmolyte, which is largely accumulated under osmotic stress in the plants' cytoplasm; the P5CS gene, which limits the speed of the proline pathway and causes the accumulation of large quantities of proline in the leaves of plants; and the rice *OsMYB4* gene, which also drives the accumulation of osmolytes, such as osmotin, have all been selected for the development of a variety of bioengineered plants in an effort to improve the given plant variety's stress-tolerance traits. Bioengineered kiwifruit plants over-expressing *AtNHX1* and bioengineered potato plants over-expressing trehalose, as well as bioengineered Carrizo citrange trees overexpressing P5CS (Molinari et al., 2004); and rice *OsMYB4* overexpressing apple trees (Pasquali et al., 2008), have all shown improved tolerance to osmotic stresses. Plants respond to cold, biotic, and other stresses by over-expressing several types of defense proteins. Osmotin is involved in apoptosis via the RAS2/cAMP pathway (Grenier et al., 1999) and the osmotin gene, which expresses the defense protein osmotin, was identified in plants responding to salt, cold and other stresses (Zhu et al., 1996). Results from field trials performed with the transgenic osmotin olive trees, cv Canino, (Rugini et al., 2008), D'angeli and Altamura, (2007) showed that the osmotin protein is positively involved in the induction of the cell death program (PCD), that becomes activated during periods of acclimatization induced by cold stress enhancing its role as a cryoprotectant by blocking the cold-induced calcium signaling, and controlling the cytoskeleton in response to cold stimuli. Other plant proteins, such as 1, 3, endoglucanases, chitinase, and thaumatin, have also demonstrated anti-freeze properties, as well as *in vivo* antimicrobial properties. Pathogenesis-related proteins (PR proteins), such as osmotin, are plants' first line of defense, and some of them have shown to exert antiviral and antibacterial activities. Genes related to plant defense have been extensively described by Grenier and Gribaudo (2012) and Rugini, (2015).

The Osmotin protein is a homologue of the human hormone adiponectin, (Narasimhan et al., 2005), which exerts its effects through the Adipor1 and Adipor2 receptors (Yamauchi, et al 2003) in mammals. In fact, adiponectin has been shown to play a major role in metabolic and regenerative processes in mammals, leading to its identification as a potential target for the development of therapeutic approaches for the treatment of diabetes and obesity (Narasimhan et al., 2005), and closely associated with the inhibition of the proliferation of a variety of cancers (Iwabu et al., 2015). Evidence has continued to mount, expanding the scientific community's understanding of the novel therapeutic functions and potential applications of the osmotin protein, and the key mechanisms through which it operates to activate regenerative processes in mammalian systems, exert neuroprotective effects and even cross the Blood-Brain Barrier (BBB) to reverse neurological diseases such as dementia, Parkinson's and Alzheimer's (Amin Fu et al., 2017).

The development of the osmotin overexpressing kiwis and olives represents an ideal answer to the growing need for novel cultivars and plant varieties capable of withstanding the major stresses facing agriculture. These plants also represent the perfect bioreactors for the mass-production of the osmotin protein, for therapeutic purposes, in line with US FDA guidance (Kwon and Daniell, 2016).

Here we describe the data obtained in laboratory experiments aimed at identifying biotic and abiotic stress tolerant olive and kiwi plants, and, in particular, plants with enhanced drought- and salt-resistance, and, furthermore, we compare such data to the tests carried out in the field. Future goals include the establishment of osmotin overexpressing olive and kiwi orchards in various soils, including ones stricken by dry-land salinity, for the cultivation of fruit-bearing trees and vines, overexpressing the osmotin protein to achieve yield stability and a sustainable source of the valuable protein osmotin for its use in the production of novel, powerful and

unique nutraceutical products and functional foods for the extension of human health span and lifespan.

2.Materials and Methods

2.1.Plant material and field trials: The experiments have been carried out both in authorized field trials for drought stresses on ten-years-old plants and for resistance to fungal diseases by artificial inoculation of solid medium discs with active growing mycelium of *Botrytis cinerea* on surface of leaves of young potted plants and on epidermis of ripened fruits also with *Cadophora luteo-olivacea* (Rugini et al., 2011; Rugini et al., 2016). A test on the olive has been performed in potted plants in controlled conditions with low water supply during dry season. In addition for in vitro tests, the drought stress was induced on shoots by using PEG 8000 (Polyethylene glycol). Both, kiwi and olive transgenic plants derived from an *Agrobacterium tumefaciens*-mediated transformation, strain LBA 4404, plasmid pK YLX71, carrying the tobacco *osmotin* gene and *NPTII* gene, under control of *35S* promoter (Rugini et al., 1999). They were obtained from leaf disc organogenesis of cv Hayward (Rugini et al. ,1991, 1999) and from somatic embryos derived from leaf petioles of cultivar Canino, through a "double regeneration technique" (Rugini and Caricato 1995; Rugini and Silvestri 2016; Rugini et al. 2016). For *in vitro* experiments, the shoots derived from buds were collected from plants in the field just before they were extirpated. The presence of transgenes in both olive (Canino *AT-17)* and kiwi (Hayward *osm 166)* shoots were verified again before testing, as reported by Silvestri et al., 2017.

2.2. Growing conditions and plant growth parameters: For olive, ten single node explants were sub-cultured in three 250 ml-jars each containing 50 ml proliferation OM medium (Rugini 1984), supplemented with three concentrations of PEG (0, 1, 2 and 4 %). Kiwi experiments have been performed using three 500 ml-jars containing fifteen single node explants on 100 ml of solid media consisting of MS (Murashige and Skoog, 1962), sucrose 3%, BAP 1 mg/L, NAA 0.01 mg/L and Plant Agar 0.6%, and supplemented with PEG 8000 (0, 1,2 and 3 %). The pH of all media has been adjusted to 5.8 before autoclaving for 20 minutes. At the end of the experiments, basal callus fresh weight of kiwi, shoot length and node numbers were measured, and dry weight were determined by heating at 105±2°C until constant weight.

2.3. Proline content: Sample of freshly harvested leaf samples (100 mg) were collected and proline concentration was determined by spectrophotometer according to Bates et al. (1973).

2.4. Enzymes from whole shoot extracts: ROS-scavenging enzymes were determined in total crude shoots (0.5 g fresh tissues) extracts according to Santangelo et al. (2003).
Guaiacol peroxidase (POD) activity (E.C. 1.11.1.7) was measured spectrophotometrically at 470 nm using guaiacol as hydrogen donor (Santangelo et al., 2003). Catalase (CAT) activity (E.C. 1.11.1.6), was evaluated by measuring the decrease in absorbance at 240 nm due to decomposition of H_2O_2 (Santangelo et al., 2003). All reported enzyme activities were linear with time and proportional to the amount of extract used. Protein content was estimated according to Bradford (1976), using BSA as standard.

2.5. Determination of malondialdehyde content: The level of lipid peroxidation was expressed as malondialdehyde (MDA) content and was determined as TBA reactive metabolites according to Astolfi et al. (2005). Fresh shoot tissues (0.2 g) were homogenized in 10 ml of 0.25% TBA made in 10% TCA. The level of lipid peroxidation was expressed as mol g^{-1} fresh

weight by using an extinction coefficient of 155 mM cm^{-1}.

2.6.Statistical analysis: Each reported value represents the mean ± SD of measurements carried out from three independent experiments. All the data were statistically analyzed using one-way ANOVA with the GraphPad InStat Program (version 3.06). Significant differences were established by posthoc comparisons (Fisher's LSD) at $P<0.01$.

3.Results and Discussions

Field trials: In field trials the transgenic kiwi lines, without any water supply during dry season (year 2012, July) characterized by heavy drought, the transgenic plants included Hay *osm166* line subjected to laboratory experiments, and showed turgid leaves in contrast to control plants (Hay *wt*) (Figure 1d), which showed heavy symptoms of stress. Similar results were obtained from potted olive plants under controlled conditions with very low water supply, in which the transgenic plants survived while the control ones died (Figure 1c).

Concerning resistance to fungal diseases, in tests carried out on kiwi fruits by mycelium inoculation with *Botrytis cinerea* and *Cadophora luteo-olivacea* on ripe fruit epidermis, the transgenic fruits resulted significantly less rotten as compared to control fruits, as described in previous work (Rugini et al., 2011) (Figure 1b). Test on young kiwi potted plans with discs of agar containing mycelium of *Botrytis cinerea* placed on leaves, after 3 weeks from artificial infection, the leaves of transgenic plants limited the damage by spot necrosis, while the leaves of the control plants were invaded by the fungus and dropped. Transgenic olive potted plants, grown along with control plants in the same environment, showed lower susceptibility to natural infection of *Spilocaea oleagina*, as demonstrated by fewer and smaller spots on the leaves as compared to control (Rugini et al., 2008).

***In vitro* tests:** Both the transgenic line Canino *AT17-1* and Hayward *osm166*, following 20 years from their constitution, still showed *in vitro* resistance to kanamycin (150 mg/L), and PCR analysis confirmed the presence of the target gene in shoots, allowing us to use them for tests.

Effect of PEG on *in vitro* shoot growth: In the control medium (0% PEG), the Canino *wt* showed significantly higher shoot length and node number compared to transgenic Canino *AT17-1*, while minor difference in terms of mean internode length were found (Table 1). Both the mean of shoot length and node number of Canino *wt* resulted drastically reduced even at the lowest PEG concentration. Similar results have been observed in kiwi *wt*: the shoot number and node number were decreased with the increase of PEG concentration, in contrast to kiwi transgenic line *osm166* (Table 2), which did not result in significant difference between shoot number and node number, while showing only a reduction of the mass of basal callus at the higher doses. The *wt* shoots of both species showed several necrotic symptoms on leaves proportionally to PEG concentrations.

Proline content: The proline contents in the shoots of Canino *AT17-1* were greater than in those of Canino *wt*, even in the control conditions (absence of PEG); these events were not observed in kiwi shoots. The shoots of Canino *wt* showed a significant increase in proline content in the presence of PEG 1 and 2%, while the further increase to 4%, resulted in a decrease of proline accumulation. Similar behaviour was observed in transgenic kiwi *osm166* (Table 2). In contrast, in transgenic olive the increase in PEG concentration in the medium was not followed by an increase of proline content in the shoots of the transgenic line at 1 and 2% of PEG, except at the highest concentration (PEG 4%); in transgenic kiwi proline content increased from the lowest

concentration, but at the highest one (3%) was similar to control (0% PEG) probably because the stress was too high for this species which notoriously is very sensitive to drought , although lower concentrations in respect to olive were used.

Total soluble protein content and antioxidant enzyme activity: No significant differences in shoot protein concentration have been observed in both *wt* and transgenic line of both species upon PEG treatment (Table 2). However, several differences were evident between the wt and transgenic lines of both species when antioxidant enzyme were considered (Table 2). Under control conditions, the basal activity of both enzymes was clearly higher in Canino *wt* leaves than in those of Canino *AT17-1*. On the other hand, PEG treatments differently affected both peroxidase and catalase activity. In Canino *wt* shoots, peroxidase activity of PEG-treated plants was significantly higher than that of the control (0% PEG) and in particular gradually increased with increasing PEG concentration from 1 to 4%. On the other hand, in Canino *AT17-1* shoots, peroxidase activity remained stable, irrespective of PEG concentration, to values similar to those of control plants. Consequently, we observed that application of the highest PEG concentration (4%) resulted in peroxidase activity in Canino *wt* shoots, more than fivefold higher than those found in Canino *AT17-1* leaves. On the other hand, catalase activity was significantly induced in all PEG exposure experiments, with concentrations ranging from 1 to 4%, in transgenic shoots, while it was almost unaffected by PEG treatment in control shoots. The experiments with kiwi genotypes confirmed what we have observed in olive, but the catalase activity did not differ between kiwi transgenic line and control plants.

Lipid peroxidation: As shown in Table 2, under control conditions (0% PEG), MDA content was significantly higher (30%) in Canino *wt* shoots than in those of Canino *AT17-1*. Furthermore, MDA content was increased by 20% following the application of the highest PEG concentration in Canino *wt* shoots, while it did not respond to PEG-exposure in leaves of the transgenic line.
In kiwi, under control conditions (0% PEG), MDA contents did not differ between Hayward *wt* and Hayward *osm166*. Similarly, with olive experiments, MDA content was increased following the application of the highest PEG concentration in Canino *wt* shoots, while it did not respond to PEG-concentration in leaves of the transgenic lines.
Our results showed the stability of osmotin gene in the plants also after about 20 years of their establishment and the over expression of this gene protects the plants from drought stress and fungal diseases. There is evidence that the presence of tobacco-osmotin gene in transgenic olive and kiwi shoots induces an accumulation of proline in the tissues that confers tolerance to osmotic stress. The significantly higher accumulation of proline in plants exposed to drought stress most likely indicates a reaction to tissue damage and according to Aghaz et al. (2013) it could be considered as an attempt for plants to reduce the oxidative damage, and according Wei et al. (2009) the drought-induced accumulation of proline could be attributed to a down-regulation of proline dehydrogenase. Under *in vitro* condition in both kiwi and olive, the transgenic line appeared less affected by the presence of PEG than the *wt* ones, whereas the leaves and shoots of wild type counterpart resulted severely affected by the stress induced by PEG. Since drought represents a major abiotic stress affecting the plant photosynthetic activity, it also adversely decreases plants' carboxylation capacity and the related energy dissipation (Wilhelm and Selmar 2011). Under these conditions, an increase in the production of reactive oxygen species (ROS) clearly occurs (Schwanz and Polle 2001; Vranovà et al. 2002), and their accumulation is commonly associated with promotion of lipid peroxidation, which contributes to membrane degradation. In particular, the MDA concentration in plant tissues is considered a marker of the degree of membrane damage resulting from ROS (Wang et al. 1999), so this finding could suggest that the transgenic lines of both species were more efficient in the

activation of defense responses against oxidative stress in respect to the *wt* ones.
The development of protective mechanisms is a crucial step to cope with oxidative stress. One of these mechanisms is the induction of antioxidant machinery, which involves the functioning of a number of enzymes such as superoxide dismutase, catalase, guaiacol peroxidase and ascorbate peroxidase, which allow elimination of excessive ROS accumulation (Shah et al. 2001).
In this study, we evaluated the general antioxidant status of the leaves from Canino *wt* and transgenic line Canino *AT17-1* plants by measuring changes in peroxidase and catalase activity profiles following PEG treatments and demonstrated significant difference in the response to drought for both enzymes.

4. *Conclusion*

This work confirms the efficacy of the transgenic approach for improving drought tolerance in olive and kiwi cultivars, and offers an early *in vitro* screening tool for the selection of drought-tolerant plant materials, as shown by the data on PEG, which conforms with the data collected in *in vivo* drought tolerance tests and, in the field in the case of kiwi plants and in potted plants in olive. In addition, we believe that the *in vitro* test operated by D'Angeli and Altamura (2007) for resistance to frost, by using the same transgenic olive Canino *At-17*, could be another method to evaluate resistant plants to frost at an early stage of growth, and just as the inoculation of mycelium in very young potted kiwi plants (Rugini et al., 2011; Rugini, 2012) could represent another early screening technique for the identification of fungal disease resistant fruits. Finally, also the observation of the content of intracellular osmotin protein in the leaves of young olive plants reported by Rugini et al. (2000) could be another precocious method for screening plants resistant to *Spilocea oleagina*.
The opportunity to introduce such plants into the market must be carefully considered for both the production of fruits, as well as for medicinal uses by consuming the lyophilized leaves directly as therapeutic food as US FDA guidance.

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5.References

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Table 1: Growth parameters of Canino *wt*, Canino *AT17-1*, Hayward *wt* and Hayward *osm166*. Data are the means (± SD) from three experiments. Different letters above the data indicate significantly different means as determined by Fisher's LSD (P<0.01).

Clone	PEG 8000 (% w/v)	Shoot lenght (mm)	Node number	Internode length(mm)
Canino *wt*	0	20.30 **a**	20.1 **a**	0.99 **a**
Canino *wt*	1	11.44 **b**	14.7 **b**	0.81 **b**
Canino *wt*	2	6.70 **c**	11.7 **b**	0.64 **c**
Canino *wt*	4	3.91 **d**	6.6 **c**	0.57 **c**
Canino *At17-1*	0	11.57 **a**	14.5 **a**	0.79 **a**
Canino *At17-1*	1	9.03 **b**	12.4 **b**	0.73 **ab**
Canino *At17-1*	2	7.05 **b**	10.5 **b**	0.67 **ab**
Canino *At17-1*	4	6.15 **b**	4.0 **b**	0.57 **b**
Clone	**PEG 8000 (% w/v)**	**Shoot number**	**Node number**	**Callus formation (g)**
Hayward *wt*	0	1.67	5.11 **a**	0.25 **a**
Hayward *wt*	1	1.56	3.67 **b**	0.14 **b**
Hayward *wt*	2	1.68	3.88 **b**	0.11 **b**
Hayward *wt*	3	1.75	3.77 **b**	0.11 **b**
Hayward *osm166*	0	1.84	6.33 **a**	0.36 **a**
Hayward *osm166*	1	1.81	6.70 **a**	0.23 **ab**
Hayward *osm166*	2	1.72	5.44 **a**	0.12 **b**
Hayward *osm166*	3	1.76	5.67 **a**	0.14 **b**

Table 2: Proline content, protein content, POD & CAT activity and MDA content in shoots of olive (Canino *wt* and Canino *AT17-1*) and kiwi (Hayward *wt* and Hayward *osm166*) lines, grown *in vitro* on media supplemented with PEG 8000. Data are the means (± SD) from three independent experiments. Different letters among the same columns within the groups indicate significantly different means as determined by Fisher's LSD at P<0.01.

Clone	PEG 8000 (% w/v)	Proline content (µg g^{-1} fw)	Protein content (mg g^{-1} fw)	POD (ΔA_{470} min^{-1} mg^{-1})	CAT ($\Delta A240$ min^{-1} mg^{-1})	MDA (µg g^{-1} fw)
Canino *wt*	0	92.8 **a**	6,796	5.31 **a**	2.24 **a**	0.0068 **a**
Canino *wt*	1	221.1 **b**	6,459	10.68 **b**	2.22 **a**	0.0065 **a**
Canino *wt*	2	233.6 **b**	7,122	13.32 **c**	2.22 **a**	0.0062 **a**
Canino *wt*	4	100.1 **a**	6,512	14.02 **c**	1.88 **a**	0.0080 **b**
Canino *At17-1*	0	570.2 **a**	6,973	3.34 **a**	0.71 **a**	0.0052 **a**
Canino *At17-1*	1	620.8 **a**	7,016	2.80 **a**	3.21 **b**	0.0044 **a**
Canino *At17-1*	2	637.0 **a**	7,791	2.64 **a**	3.27 **b**	0.0048 **a**
Canino *At17-1*	4	1029.3 **b**	7,362	2.48 **a**	3.33 **b**	0.0048 **a**
Hayward *wt*	0	23.2 **a**	7,861	19.81 **a**	1.01	0.0029 **a**
Hayward *wt*	1	24.2 **a**	7,324	24.34 **b**	0.99	0.0032 **a**
Hayward *wt*	2	22.1 **a**	6,588	29.29 **bc**	0.84	0.0033 **a**
Hayward *wt*	3	19.8 **a**	7,245	36.60 **c**	0.79	0.0048 **b**
Hayward *osm166*	0	23.2 **a**	7,444	18.84 **a**	1.17	0.0032 **a**
Hayward *osm166*	1	55.3 **b**	7,661	16.12 **a**	0.84	0.0029 **a**
Hayward *osm166*	2	58.4 **b**	7,161	15.89 **a**	0.85	0.0030 **a**
Hayward *osm166*	3	25.1 **a**	6,923	9.69 **b**	0.84	0.0029 **a**

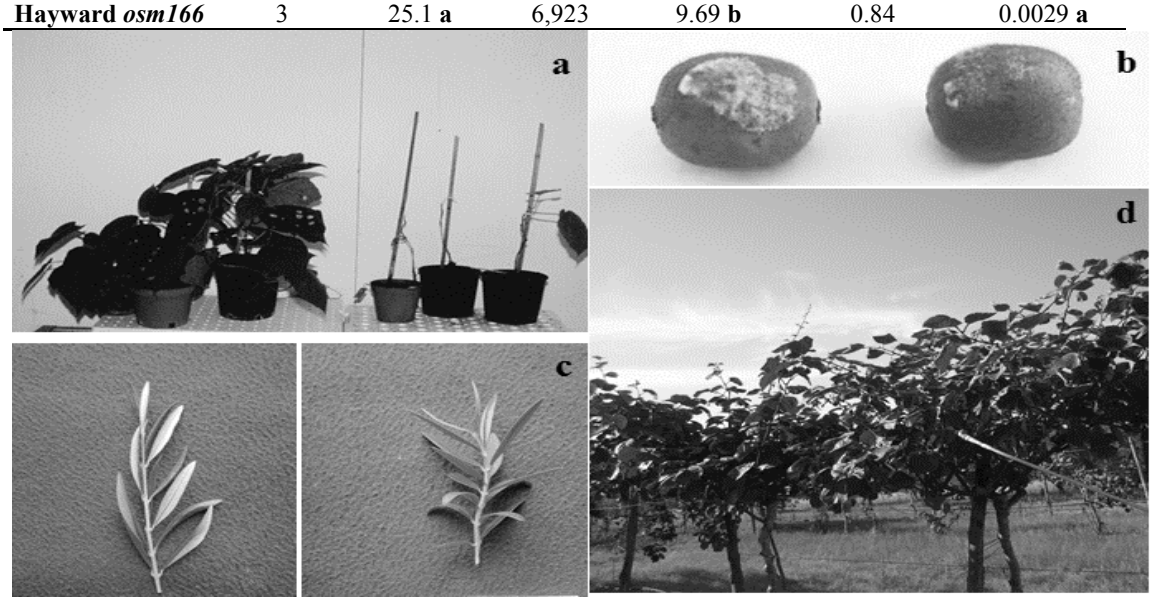

Figure 1. (a) *Botrytis cinerea* test on kiwi plants: discs of agar with mycelium placed on leaves of young potted kiwi plants after 3 weeks from artificial infection. The leaves of transgenic plants (left group) limited the damage by spot necrosis, while the leaves of the control plants (right group) were invaded by the fungus and dropped. (b) *Botrytis cinerea* test on ripened kiwi fruits. Note the large area of mycelium on the surface of Control fruit (left) making rot the low tissues; the transgenic fruit (right) drastically reduced the mycelium proliferation and rot. (c) Apical shoots of olive detached from two and half years potted plants watered with minimum dose of water during summer time. Note the still growing and well hydrated transgenic with osmotin gene shoot (right), contrary to control shoot which appears completely dry and brownish in color. (d) Two contiguous kiwi plants growing in authorized field trial, in July without irrigation. The control plants (right) started to dry and leaves are pale green, while the transgenic plant (left) is well hydrated with dark green leaves.