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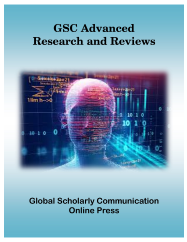

(RESEARCH ARTICLE)

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Trichoderma viride inoculated in the growing medium for the vitamin C increase in the leaves of *Kalanchoe spp.* and defense against *Pithyum sp.*

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Abstract

The article presents the results of research on succulents such as *Kalanchoe pinnata*, *Kalanchoe tubiflora* and *Kalanchoe gastonis-bonnieri*, aimed at improving plant growth and defense against the pathogenic fungus *Pythium sp.*, through the use of *Trichoderma viride* inoculated in the medium. Objectives of the work were: 1) use *Trichoderma viride* to assess whether the use of this endophytic fungus can increase the growth rate of *Kalanchoe pinnata*, *Kalanchoe tubiflora* and *Kalanchoe gastonis-bonnieri*, plants generally slow in their growth cycle; 2) evaluate if using *Trichoderma viride* can lead to an increase in vitamin C content in the leaves of *Kalanchoe spp.*; 3) assess and evaluate how the use of *Trichoderma viride* allows greater protection of the roots from *Pithyum sp.* which often affects the roots of these succulents. The two experimental groups in cultivation were: i) group without *Trichoderma spp.*, irrigated with water and substrate previously fertilized; ii) group with *Trichoderma viride*, irrigated with water and substrate previously fertilized.

The test showed a significant increase in agronomic parameters analyzed in plants treated with *Trichoderma viride.* In fact, all plants treated showed a significant increase in height and number of leaves per plant, vegetative and root weight of plants, number and weight of new shoots, total number of germinated seeds/ 100 seeds sown and a significant reduction in average germination time. In addition, plants treated with Trichoderma show an increase in leaf vitamin C content, dry mass and a reduction in plant mortality due to *Pythium sp.*

Tricoderma can also optimize the use of fertilizers and water by facilitating the cultivation of plants in certain locations subject to biotic and abiotic stress.

Keywords: Sustainable Applications; *Kalanchoe Pinnata; Kalanchoe Tubiflora; Kalanchoe Gastonis- Bonnieri; Trichoderma Spp.*

1. Introduction

Family Crassulaceae is a large family of dicotyledons, composed mainly of succulent herbs and shrubs or miniature trees in some genera and species. Most family members have the ability to store water in the leaf and stem [1,2]. Some are believed to be able to absorb water directly from the air through special hairs, epidermal cells or adventitious roots [3]. The members of this family are not considered important growing plants, but are used for horticulture; many members have an unusual and attractive appearance, and are quite resistant, usually need minimal care [4].

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Kalanchoe juice is used for local treatment of tooth and mouth disease, wounds, insect bites, fever, respiratory and skin diseases, cholera, urinary diseases, tissue diseases, arthritis and stomach ulcers. Crushed leaves can be used for headaches, rheumatism, lung, immune and gastric diseases [5,6,7,8,9]

The genus Trichoderma consists of anamorphic fungi isolated primarily from soil and decomposing organic matter, with teleomorphs, when known, belonging to the ascomycete genus Hypocrea (order Hypocreales). Fungal species belonging to this genus are worldwide in occurrence and easily isolated from soil, decaying wood and other plant organic matter. Trichoderma isolates are characterized by a rapid growth rate in culture and by the production of numerous spores (conidia) with varying shades of green. Their lifestyle is generally saprotrophic with minimal nutritional requirements; they are able to grow rapidly on many substrates, can produce metabolites with demonstrable antibiotic activity and may be mycoparasitic against a wide range of pathogens [10]. The abundance of *Trichoderma spp*. in various soils, coupled with a wide metabolic versatility, a dynamic colonization of plant rhizosphere and the ability to antagonize and repress a great number of plant pathogens are direct evidence of the role that these fungal species may play in biological control [11,12]. A number of isolates of Trichoderma have been found to be effective biocontrol agents of various soil-borne plant pathogenic fungi under greenhouse and field conditions. The knowledge of mechanisms of interaction of *Trichoderma spp.* with plant pathogenic fungi and the plant host is of importance to enhance the practical application of these beneficial microorganisms. They can work against fungal phytopathogens either directly through mechanisms such as mycoparasitism, competing for nutrients and space, modifying environmental conditions and antibiosis or indirectly promoting plant growth and plant defensive mechanisms. For all these reasons, the use of *Trichoderma spp.* strains as inoculants of substrates to be employed in nursery could confer an additional value both in order to control soilborne pathogens, to induce resistance or to promote growth of plants.

In this experiment, the main objective was to:

1) Use *Trichoderma viride* to assess whether the use of this endophytic fungus can increase the growth rate of *Kalanchoe pinnata*, *Kalanchoe tubiflora* and *Kalanchoe gastonis-bonnieri*, plants generally slow in their growth cycle;

2) Evaluate if using *Trichoderma viride* can lead to an increase in vitamin C content in the leaves of *Kalanchoe spp*.;

3) assess and evaluate how the use of *Trichoderma viride* allows greater protection of the roots from *Pithyum sp.* which often affects the roots of these succulents.

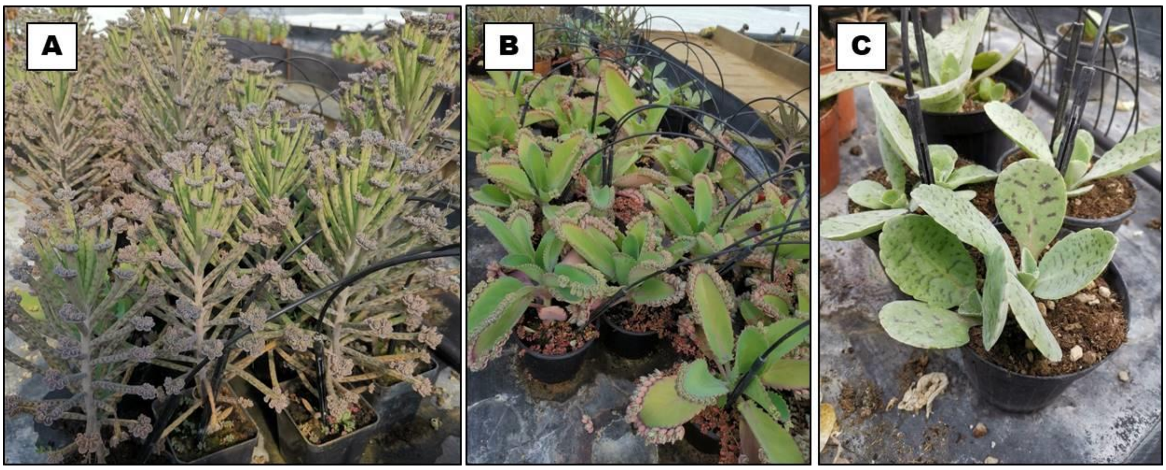

Figure 1 detail of *Kalanchoe tubiflora* (A), *Kalanchoe pinnata* (B) and Kalanchoe Gastonis-bonnieri (C) in greenhouse

2. Material and methods

2.1. Greenhouse experiment and growing conditions

The experiments, started in January 2020, were conducted in the greenhouses of CREA-OF in Pescia (Pt), Tuscany, Italy (43°54′N 10°41′E) on *Kalanchoe tubiflora, Kalanchoe pinnata* and *Kalanchoe gastonis-bonnieri* (Figure 1A,1B,1C)

The plants were placed in ø 12 cm pots; 60 plants per thesis, divided into 3 replicas of 30 plants each. All plants were fertilized with a controlled release fertilizer (2,5 kg m^{-3} Osmocote Pro®, 6 months with 190 g/kg N, 39 g/kg P, 83 g/kg K) mixed with the growing medium before transplanting.

The two experimental groups in cultivation were:

i) group without Trichoderma spp. (CTRL) (peat 50% + pumice 30%+sand 10%+zeolite 10%), irrigated with water and substrate previously fertilized;

ii) group with *Trichoderma viride* (TV) (peat 50% + pumice 30% + sand 10%+zeolite 10%), irrigated with water and substrate previously fertilized. *Trichoderma viride* has been isolated by TNC Mycorr MAX (1.3 $x10^8$ cfu/Kg). Dosage: 50g into 15 litres of substrate.

The plants were watered 2 times per week and grown for 10 months. The plants were irrigated with drip irrigation. The irrigation was activated by a timer whose program was adjusted weekly according to climatic conditions and the fraction of leaching. On October 15, 2020, plants height, leaves number, vegetative and radical weight, number and weight of new shoots, seed germination and average germination time were recorded. Additionally, in the experiment the content of vitamin C in the leaves of *Kalanchoe pinnata*, *Kalanchoe tubiflora* and *kalanchoe gastonis-bonnieri* and the presence of plant mortality following attacks of *Pithyum sp.* was evaluated.

2.1.1. Statistics

The experiment was carried out in a randomized complete block design. Collected data were analysed by one-way ANOVA, using GLM univariate procedure, to assess significant ($P \leq 0.05$, 0.01 and 0.001) differences among treatments. Mean values were then separated by LSD multiple-range test ($P = 0.05$). Statistics and graphics were supported by the programs Costat (version 6.451) and Excel (Office 2010).

3. Results

3.1. Plant growth

The test showed a significant increase in agronomic parameters analyzed in plants treated with *Trichoderma viride* on *Kalanchoe pinnata*, *Kalanchoe tubiflora* and *Kalanchoe gastonis-bonnieri*. In fact, all plants treated with *Trichoderma viride* (TV) showed a significant increase in height and number of leaves per plant, vegetative and root weight of plants, number and weight of new shoots, total number of germinated seeds/ 100 seeds sown and a significant reduction in average germination time. In addition, plants treated with Trichoderma show an increase in leaf vitamin C content, dry mass and a reduction in plant mortality due to *Pythium sp.*

Particularly in *Kalanchoe pinnata* (Table 1), treatment with *Trichoderma viride* (TV) significantly improved plant height, 15.58 cm (TV) compared to 13.54 cm (CTRL) (Figure 2B). It significantly increased the number of leaves per plant by 20.40 (TV) compared to 15.00 (CTRL) of the untreated control. There was also an increase in vegetative weight, 46.06 g (TV) compared to 42.94 g (CTRL) (Figure 3B) and root weight 33.04 g (TV) compared to 28.44 g of the control. The test also showed a significant increase in the number of new shoots 89.00 (TV) compared to 79.40 (CTRL), shoots weight 4.10 (GM) compared to 3.18 (CTRL), total number of germinated seeds/100 seeds, 73.80 (TV) compared to 65.60 of the untreated control. There was also a reduction in the average germination time, 16.60 days in (TV) compared to 22.40 days in the control (CTRL).

Similarly in *Kalanchoe tubiflora* (Table 2), treatment with *Trichoderma viride* (TV) showed a significant increase in plant height of 25.40 cm, compared to 23.12 cm in control (CTRL) (Figure 2A). The Trichoderma treatment also improved leaf count, 41.20 (TV) compared to 32.80 (CTRL). There was also a significant increase in vegetative weight, 37.72 g (TV) compared to 35.90 g (CTRL) and root weight 27.84 g (GM) compared to 23.80 g of the untreated control. There was also a significant increase in new shoots 129.60 (TV) compared to 114.80 (CTRL), shoots weight, 3.18 (TV) compared to 2.21 of the control and total number of seeds germinated, 84.20 (TV) compared to 76.00 of (CTRL). In addition there was a significant reduction in average germination time, 18.00 days in (TV) compared to 22.80 days of the control.

In *Kalanchoe gastonis-bonnieri* (Table 3), plants treated with *Trichoderma viride* showed a significant increase in plant height of 14.06 cm (TV), compared to 10.20 cm (CTRL). There was also a significant increase in leaf count, 24.40 (TV) compared to 20.42 (CTRL). Even on *K. gastonis-bonnieri* there was an increase in vegetative weight 44.86 g (TV) compared to 42.32 g (CTRL) and in root weight 31.18 g (TV) compared to 28.40 g (CTRL) (Figure 4). It was also evident the significant increase in the number of shoots in plants treated with Trichoderma, 73.80 (TV) compared to 64.22 (CTRL), the increase in the weight of shoots 4.82 g in (TV) compared to 4.34 in the untreated control (Figure 3A), the total number of seeds germinated/100 seeds sown, 81.41 (TV) compared to 74.83 in the control. Also in this case there was a reduction of the average germination time in (TV), 13.61 days compared to 16.86 days in the control.

The test also showed that the use of *Trichoderma viride* in the growing medium can increase the vitamin C content in the leaves, the dry mass percentage and the control of plant mortality due to *Pythium sp.* on *K. pinnata*, *K. tubiflora* and *K. gastonis-bonnieri.* In *K. pinnata* (Table 4), plants treated with Trichoderma showed a significant improvement in the vitamin C content of the leaves, 74.81 mg in (TV) compared to 70.23 mg (CTRL), in the percentage of dry biomass, 9.04% (TV) compared to 7.78% (CTRL). There was also a reduction in plant mortality due to *Pythium sp.*, 1.21 (TV) compared to 6.83 (CTRL). In *K. pinnata* (Table 4), plants treated with Trichoderma showed a significant improvement in the life expectancy C content of the leaves, 74.81 mg in (TV) compared to 70.23 mg (CTRL), the percentage of dry biomass, 9.04% (TV) compared to 7.78% (CTRL). There is also a reduction in plant mortality due to *Pythium sp.*, 1.21 (TV) compared to 6.83 (CTRL). The same trend also in *K. tubiflora* with a vitamin C content of 9.21 mg (TV), compared to 7.43 mg of the control, the percentage of dry biomass in (TV) of 4.28% compared to 3.36% of (CTRL) and a reduction in plant mortality of 1.42 (TV) compared to 5.66 of the control. In *K. gastonis-bonnieri* (Table 4) the vitamin C content of leaves in (TV) was 20.66 mg compared to the control of 13.58 mg, the percentage of dry biomass, 7.65% (TV) compared to 6.54% of the control. Plant mortality was also 0.64 (TV) compared to 3.47 in the control (CTRL).

Table 1 Evaluation of *Trichoderma viride* on agronomic characters on plants of *Kalanchoe pinnata*

Groups	PH (cm)	LN (n°)	VW (g)	RW (g)	NSN (n°)	NSW (g)	SG/100seed (n°)	TG (days)
CTRL	13,54 b	15,00 b	42,94 b	28,44 b	79,40 b	3,18 b	65,60 b	22,40 a
TV	15,58 a	20,40 a	46,06 a	33,04 a	89,00 a	4,10 a	73,80 a	16,60 b
ANOVA	**	***	***	***	***	***	**	***

One-way ANOVA; n.s. – non significant; *,**,*** – significant at P ≤ 0.05, 0.01 and 0.001, respectively; different letters for the same element indicate significant differences according to Tukey's (HSD) multiple-range test (P = 0.05). Legend: (CTRL) control; (TV) *Trichoderma viride;* PH: plant height; LN: leaves number; VW: vegetative weight; RW: roots weight; NSN: new shoot number; NSW: new shoot weight; SG/100seed: total germination on 100 seeds; TG: time germination

Table 2 Evaluation of *Trichoderma viride* on agronomic characters on plants of *Kalanchoe tubiflora*

Groups	PH (cm)	LN (n°)	VW (g)	RW (g)	NSN (n°)	NSW (g)	SG/100seed (n°)	TG (days)
CTRL	23,12 b	32,80 b	35,90 b	23,80 b	114,80 b	2,21 b	76,00 b	22,80 a
TV	25,40 a	41,20 a	37,72 a	27,84 a	129,60 a	3,18 a	84,20 a	18,00 b
ANOVA	**	***	**	***	***	***	***	***

One-way ANOVA; n.s. – non significant; *,**,*** – significant at P ≤ 0.05, 0.01 and 0.001, respectively; different letters for the same element indicate significant differences according to Tukey's (HSD) multiple-range test (P = 0.05). Legend: (CTRL) control; (TV) *Trichoderma viride;* PH: plant height; LN: leaves number; VW: vegetative weight; RW: roots weight; NSN: new shoot number; NSW: new shoot weight; SG/100seed: total germination on 100 seeds; TG: time germination

Table 3 Evaluation of *Trichoderma viride* on agronomic characters on plants of *Kalanchoe gastonis-bonnieri*

Groups	PH (cm)	LN (n°)	VW (g)	RW (g)	NSN (n°)	NSW (g)	SG/100seed (n°)	TG (days)
CTRL	10,20 b	20,42 b	42,32 b	28,40 b	64,22 b	4,34 b	74,83 b	16,86 a
TV	14,06 a	24,40 a	44,86 a	31,18 a	73,80 a	4,82 a	81,41 a	13,61 b
ANOVA	***	**	***	***	***	**	**	***

One-way ANOVA; n.s. – non significant; *,**,*** – significant at P ≤ 0.05, 0.01 and 0.001, respectively; different letters for the same element indicate significant differences according to Tukey's (HSD) multiple-range test (P = 0.05). Legend: (CTRL) control; (TV) *Trichoderma viride;* PH: plant height; LN: leaves number; VW: vegetative weight; RW: roots weight; NSN: new shoot number; NSW: new shoot weight; SG/100seed: total germination on 100 seeds; TG: time germination

Table 4 Evaluation of *Trichoderma viride* in leaves vitamin C production and protection against *Pythium* sp. in *kalanchoe pinnata, Kalanchoe tubiflora* and *Kalanchoe gastonis-bonnieri*

Groups	Kalanchoe Pinnata			Kalanchoe tubiflora			Kalanchoe gastonis-bonnieri		
	Vit.C mg/100 g of juice	Dry Mass (%)	*Pythium sp.* plants affected (n°)	Vit.C mg/100 g of juice	Dry Mass (%)	*Pythium sp.* plants affected (n°)	Vit.C mg/100 g of juice	Dry Mass (%)	*Pythium sp.* plants affected (n°)
CTRL	70,23 [b]	7,78	6,83 [a]	7,43 [b]	3,36	5,66 [a]	13,58 [b]	6,54	3,47 [a]
TV	74,81 [a]	9,04	1,21 [b]	9,21 [a]	4,28	1,42 [b]	20,66 [a]	7,65	0,64 [b]
ANOVA	*	-	***	**	-	***	**	-	***

One-way ANOVA; n.s. – non significant; *,**,*** – significant at P ≤ 0.05, 0.01 and 0.001, respectively; different letters for the same element indicate significant differences according to Tukey's (HSD) multiple-range test (P = 0.05). Legend: (CTRL) control; (TV) *Trichoderma viride*

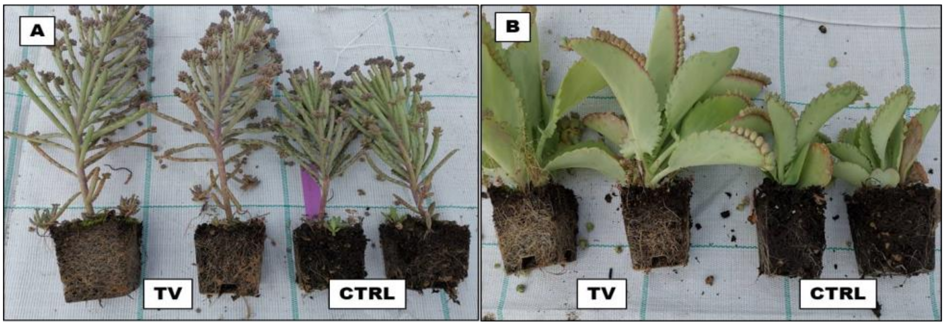

Figure 2 Effect of *Trichoderma viride* on vegetative biomass of *Kalanchoe tubiflora* (A) and *Kalanchoe pinnata* (B). Legend: (CTRL) control; (TV) *Trichoderma viride*

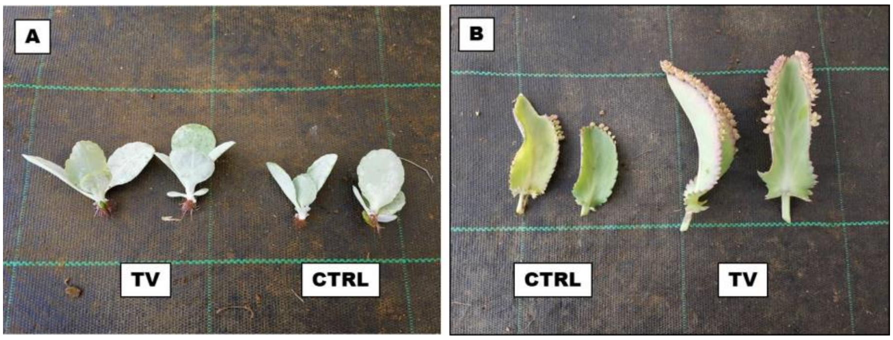

Figure 3 Effect of *Trichoderma viride* on production of new shoots in *Kalanchoe gastonis-bonnieri* (A) and leaves size in *kalanchoe pinnata* (B). Legend: (CTRL) control; (TV) *Trichoderma viride*

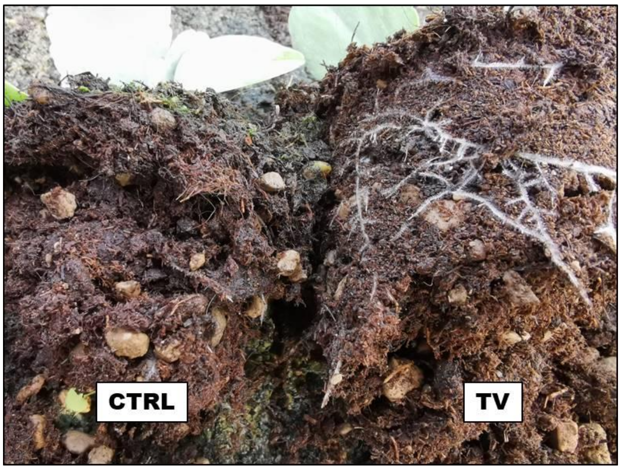

Figure 4 Effect of *Trichoderma viride* on roots biomass of *kalanchoe gastonis-bonnieri*. Legend: (CTRL) control; (TV) *Trichoderma viride*

4. Discussion

Treatments with *Trichoderma viride* have resulted in a significant improvement in plant growth characteristics, vitamin C content of leaves and protection against *Pythium sp.*, in *Kalanchoe pinnata*, *Kalanchoe tubiflora* and *kalanchoe gastonis-bonnieri*. In recent years, *Trichoderma spp*. has been widely used in agriculture both as a biocontrol agent for plant pathogens and as a plant growth promoter [13]. In fact, these fungi are involved in fundamental activities that ensure the stability and productivity of both agricultural and natural ecosystems. Some Trichoderma strains behave as endophytes, colonizing the radical epidermis and external cortical layers and releasing bioactive molecules. At the same time, the transcriptome and proteome of the plants are substantially altered [14].

This intimate fungus-plant interaction provides a number of benefits only recently recognized for their variety and importance, including increased plant resistance to various biotic stresses through induced or acquired systemic resistance and abiotic stresses such as water deficit/excess, high salinity and extreme temperature; increased efficiency in nitrogen use due to improved nitrogen reduction and assimilation mechanisms and reduced over-expression of stress genes or accumulation of toxic compounds during the plant's response to the pathogen [13]. Additional benefit from the use of Trichoderma comes from the increase in antioxidant content in fruits and vegetables grown with selected strains In addition, it has also been observed that the fertility of soils treated with certain Trichoderma strains could be significantly improved beyond disease control, which increases the attractiveness of these fungi for general use in plant production. As suggested also in this experiment the use of *Trichoderma viride* can determine an increase in vegetative and root development, as already found in other experiments in horticulture and fruit-growing where an increase in leaf surfaces, chlorophyll content and yields (size and/or number of flowers or fruits) is detected [15,16,17]. The molecular mechanisms that support this highly desirable beneficial effect of plant growth promotion are not fully understood and include improved nutrient availability and uptake for the plant [18,14]. Further analysis shows a general increase in the uptake of many elements such as Pb, Mn, Zn, Al and the ability to solubilize certain nutrients in soil, such as phosphates, Fe^{3+}, Cu^{2+}, Mn^{4+} ions, many times not readily available from the plant. [18].In addition, the involvement of both plant and fungal growth hormones could be involved in the phenomenon of plant growth promotion [19]. In combination with direct effects on plant pathogens and the ability to promote plant growth, *Trichoderma spp.* has also been found to stimulate plant defense mechanisms. In this test *Trichoderma viride* was able to control the development of *Pythium sp.* The presence of Trichoderma in plants leads to an induction of resistance, often localized or systemic [14]. This phenomenon, observed also in the field, has been attributed to a fungus-based biochemical cross talk involving many bioactive metabolites produced by biocontrol agents [13,14,20]. During the colonization process the Trichoderma hyphae wrap themselves around the roots, form structures similar to appressors and finally penetrate the root cortex. The cells of the epidermis and cortex in the root are induced to deposit cell wall material and produce phenolic compounds, which results in control over the development of Trichoderma [19]. However, some more performing strains of Trichoderma are able to induce a stronger response in the plant than the immunity triggered by the pathogen and determine the production of secondary metabolites and enzymes with antimicrobial activity and the accumulation of antimicrobial compounds and phytoalexins that play a fundamental role in the protection of plants from biotic and abiotic stress [17].

5. Conclusion

The test showed that the use of *Trichoderma viride* can improve plant growth, vitamin C content in leaves and protection from *Pythium sp.* in *Kalanchoe pinnata*, *Kalanchoe tubiflora* and *Kalanchoe gastonis-bonnieri*. In particular in treated plants we find a significant increase in height and number of leaves per plant, vegetative and root weight of plants, number and weight of new shoots, total number of germinated seeds/ 100 seeds sown and a significant reduction in average germination time. In addition, plants treated with Trichoderma showed an increase in leaf vitamin C content and dry mass. As in other experiments *Trichoderma viride* proved to be an excellent biocontrol agent, in this case against *Pythium sp.* on succulent plants. This could be a valid alternative for those growers who, during the cultivation cycle of ornamental plants, especially on cactus and succulent plants, pay attention to the quality of the plants and the reduction of the use of plant protection products released into the environment. Tricoderma can also optimize the use of fertilizers and water by facilitating the cultivation of plants in certain locations subject to biotic and abiotic stress.

Compliance with ethical standards

Acknowledgments

The article is part of the "Microsuc" project: microorganisms for the growth and protection of cacti and succulent plants

Disclosure of conflict of interest

The author declares no conflict of interest.

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