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Transmission of Protective Alert Signals, through Arbuscular Mycorrhizal, in Tomato Cultures

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Abstract – **In previous work we show that changes related with the production of high anthocyanins content (HAC) tomato plants, will produce one environmental signal able to induce disease resistance. In these work we optimize the protective signals and analyze the transmission way. Using an isolate orchard as control, we show the differences in growth, fruit production, quality and intercommunication of tomato plants. At last we analyze the number of arbuscular mycorrhiza and molecules that are involved in the plant communication, using RNA nanosondes.**

Keywords – **Arbuscular Mycorrhizal, Plant Alert Signals, Tomato Cultures, miRNA 319.**

I. Introduction

Use of mixed plant cultures to improve and optimize resistance to disease (1), is a farming strategy, that is day by day more applied. Also overexpression of some metabolic genes is a well know way to enhanced bacterial resistance of the plant itself (2, 3). These may be an explanation to observed resistance of HAC plants to usual local infections. In previous author works, (4) we show that this resistance could be extended to the surrounding plants. How to extend this resistance signal and may be optimized? This are the question that we have try to resolve here.

For signal optimization, we design a matrix of plant distribution that help transmission of protective alert signal, including control plants physically separated. All of them are cultured in orchard or isolate plot in "ecological culture conditions" according with Christian Ulrichs et al (5) and using rules of Mark Schonbeck for Cropping Systems (6). The plants are placed in a matrix of 9x9 with a 60 cm separation between them. Previous to fruit collection, plants were examined, one by one, looking for fungal or parasitic infections. Tomato plant grow, was measured periodically, and fruits are carefully examined at harvesting time looking for disease signals. Samples of roots, are sampled a tree different culture times. Root status, hyphal expansion is also examined comparing with control group. Finally, presence of a specific miRNA was measured in histological slides of rood samples.

II. Material and Methods

A. *High Anthocyanin Contends Plants*

Stable hybrids between the GMO from Norfolk Plant Sciences (Dark Indigo) (7) and a natural tomato (AKA Indigo Blue Berries) (8) has been used as high anthocyanin plants. It is a new, small. The obtained hybrid has been cultured until second generation (F2) in order to obtain a high content stable and commercial plant as described in previous work (4).

B. *Co-cultured plants.*

Tomatoes used habitually for farming in Basque Country, Jack -F1, Robin-F1 and a wide range of other tomato varieties, has been used in co-culture and as control plants.

C. *Culture area.*

One isolated culture area was prepared for de experiment. One matrix of 9x9 plants separated by 60 cm DS 10 cm, free spaces, surrounded by one line of cultured high capsaicin contends plants (Capsicum annum). Both cultures are sealed by one 1m upward stone wall.

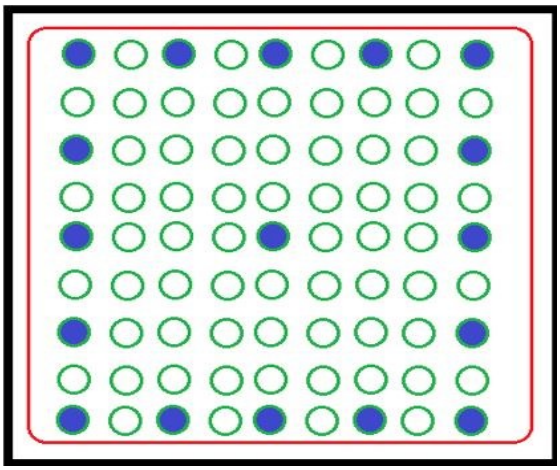

Fig. 1. Tomato plants distribution in a 9x9 matrix, red lines indicate Capsicum culture, black line stone walls. Blue dots HAC plants.

The area is oriented in a south-west position with a slope of 10º. This is mandatory in tomato cultures in Basque Country (43°19'27.6"N 1°57'39.0"W) due to proximity of limit cultivation zone. The weather in Basque Country (north of Spain) is very wet, with media relative moisture of 75 %, 19,06 ºC media temperature, 51,73 mm rain, and 10 days raining each month (9), during culture time (June, July, August). These limiting culture conditions, increase difficulty to obtain healthy tomato culture under ecological culture rules. Many parasitic and fungi diseases will appear inside cultures. Soil is clayey with calcium deficiency (10) and previous to culture starting the soil have been inoculated with commercial mycorrhiza's (Glomygel®), in order to maximize plant grow (4,11).

D. *Growth Measure and Sample Collection.*

Identification of diseases was by visual inspection, at first harvest time. Growth measure and sample collection was done at harvesting time. Height of the plants (cm.): It was carried out by means of a graduated rule from the base of the stem to the apex of the plant.

Fig. 2. A general overview of tomato culture. The first line plants are capsicum producers.

The length of external hyphae was determined after the hyphae were extracted using a membrane filter technique (12). A grid intersection method (13) was used to estimate the length of hyphae. Cores (4, 5 cm deep, 8 cm diameter) were taken at 30 cm from plant holding. Samples of soil was washed through a 250 pm mesh sieve using 500 ml of deionized water in spray. Hyphae in the soil (including those washed off roots) were thus extracted. The washings were collected and blended at high speed for 30 s in a warring blender, then transferred to a flask and stirred during the removal of 30 ml aliquots. The 30 ml aliquots were pipetted onto a Millipore filter (1 - 2 pm pore size diameter). The filter (while still in the filter holder) was covered with lactic glycerol - Trypan Blue for 5 min, rinsed with deionized water, then cut into halves and placed on microscope slides to dry. Dry filters were mounted under a coverslip and the hyphae were measured using a grid intersection method at 200 x magnification with a Dino-lite® digital microscope. Random fields of view were examined on each half filter.

E. *Protocol for measuring the degree of Arbustular Mycorrhization in plants.*

Staining of hyphae in soil: blue staining of lacto phenol is used to observe fungi. It is a simple dye (a single dye) and as such is based on the affinity of the dye for cell components, in this case for the fungal structures. Blue lacto phenol has three characteristics that make it special to observe these structures in fungi of the mould type obtained in crops by isolation. Phenol destroys the accompanying flora (sometimes in crops, bacteria can grow together with fungi). Lactic acid retains the fungal structures by creating, so to speak, a film that protects them caused by a change in osmotic gradient between the inside and the outside of the structure. Cotton blue has the ability to adhere to the hyphae and conidia of microscopic fungi. (14).

Staining and measurement of micronized roots: method given by Phillips and Hayman (15). First, roots previously alkalinized in 10% KOH were acidified in 10% HCl instead of 1% HCl as in the original procedure. Second, the concentration of trypan blue increased from 0.05% to 0.1%. The following steps are carried out:

1. Root segments of a length of approx. 0.5-1.5 cm are heated at 90 ° C in 10% KOH for 1-2 h, depending on the color and thickness of the roots.
2. The deleted root segments are rinsed in the tap water for a few minutes.
3. The root segments are heated in 10% HCl for 1 h.
4. The root segments are heated in trypan blue to 0.1% for 1-2 h.

Steps 1, 3 and 4 can be done without heating, putting the roots in KOH, HCl and trypan blue at room temperature for at least 12 h.

- Ink staining method: clarify the roots in 10% potassium hydroxide solution (m / v) incubating in an oven at 90 ° C for 30 minutes to an hour and then remove the solution, washing several times with plenty of running water. The staining solution is then added (5% Sheaffer brand ink in natural white vinegar), left to stand for 15 minutes at room temperature and then placed in the oven for 10 to 15 minutes at 70 ° C. The excess staining samples are cleaned with cold water. (16)

F. *Histopathological Analysis.*

A collection of different types of roots and hyphae from (12) different tomato plants was carried out. In both cases experimental culture and pots. They were introduced in different boats in which some were introduced formal--dehyde. To be able to do the procedure of pathological anatomy and cytology. Once the roots and hyphae have been introduced in the different canisters with formal--dehyde, they are introduced into cassettes and introduced into the processor once the procedure is finished, they are taken to the inclusion zone where we introduce the samples in the hot paraffin zone with the help of tweezers we will open the cassettes, we will look well through the cassette to not leave any pieces. We choose a mold where we will pour some paraffin with the dispenser then we will place the roots or hypha's we will place the lower part of the cassette and we will put paraffin, again from the dispenser and we will place it in the cold plate.

We start with the microtome roughing to 20 microns the different samples one by one (is rough and put back in the freezer), then begin to make the cuts to 3-5 microns which would be introduced in the bath and then take them with the slide. We would let them dry for a while and finally we would put them to the dyer to dye them. In case of making any special staining, special slides would be taken, which keep the sample in a place without moving and leave a whole day in the oven and the next day will be stained. (17)

G. In Situ Hybridization on EDC-fixed FFPE tissues using DIG-labeled miLNA™ Detection Probes.

DIG-labeled miLNA has been obtained from Exiqon ®, according with the sequence of 319 miRNAs. These miRNA, has been selected as target, due the previous works (18, 19, 20), that indicate their activity is related with tomato disease resistance and homogenous grow (21, 22, 23, 24). FFPE-ISH procedure has been done according with Exiqon ® Procedures (25, 26, 27).

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III. Results

A. *Production Protection.*

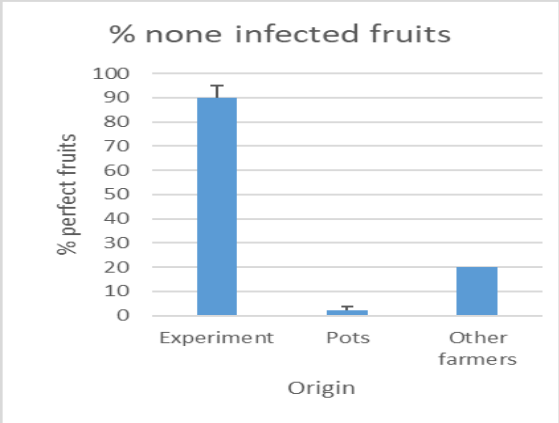

Fig. 3. Percentages of none infected Tomatoes in dependence of culture place. (n = 72 for experiment and pots, other farmers are a medium of four surrounding farmer's measures as example).

The number and quality of perfect fruits was a surprise. The area of experimentation has been used, for tomato cultures for more than twenty years and never produce a similar productivity.

B. *Plant Development.*

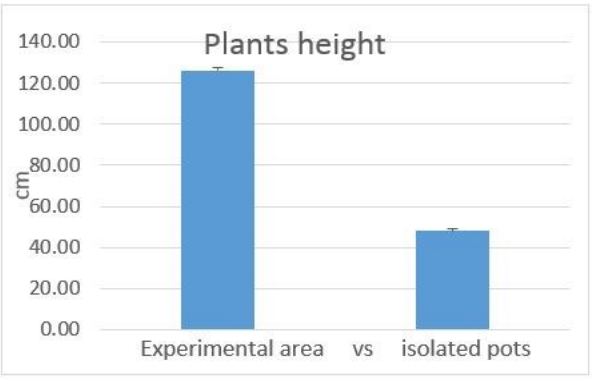

Fig. 4. Comparison of plants height (cm) in culture area vs isolated pots. n = 12.

Tomato plants in the experimental area was three times bigger than those of isolated pots at harvesting time, with a very low variability of size measures in both cases.

C. *Histological Analysis*

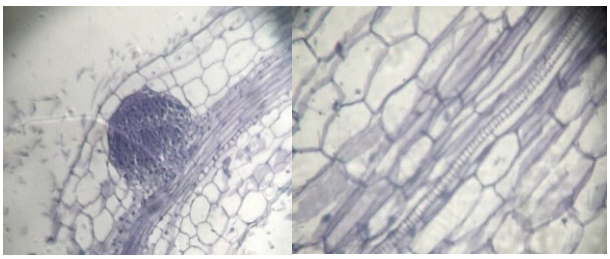

Fig. 5. Vesicle and intramatrical hypha in tomato roots. (n= 70 root samples).

Histological samples of tomato roods were processed and analyzed, as indicated in material & methods. One centimeter sections of each apical rood was observed and number of hairs, arbuscules, vesicles and extrametrical chlamidospore/ hyphas was compared between experimen--tal area and isolated pots plants.

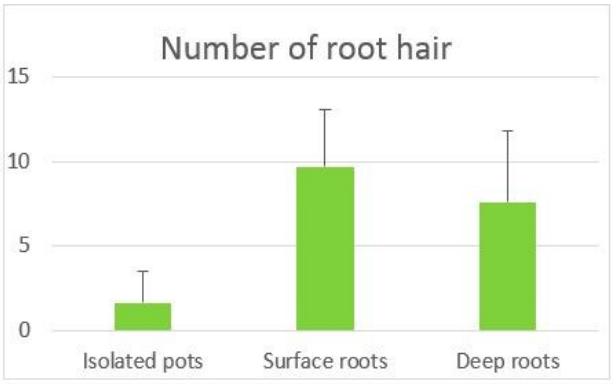

Fig. 6. Number root hair on tomato roots. (n = 70).

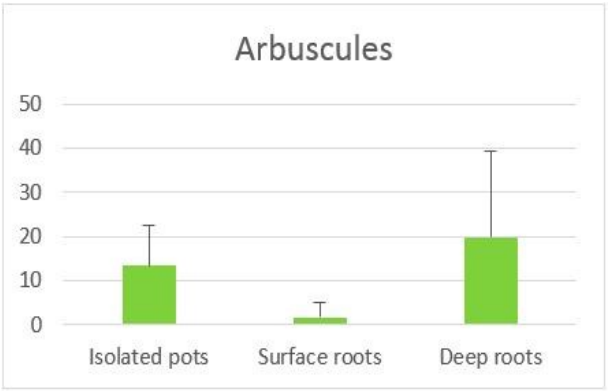

Fig.7. Number of Arbuscule & vesicles in tomato roots. (n = 70).

Superficial roots of the tomatoes in free culture, are practically without establishing a symbiotic relationship with the mycorrhizas and at a level of symbiosis equal to or lower than the isolated plants. In deep samples (30 cm depth) the number of vesicles, arbuscules and hyphaes is increased. (n = 70 samples).

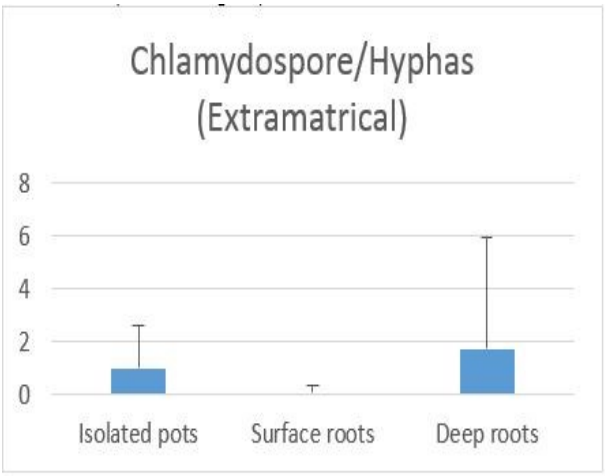

Fig. 8. Extramatrical Chlamidospore/Hyphas (n = 70).

D. *miRNA 319 Detection.*

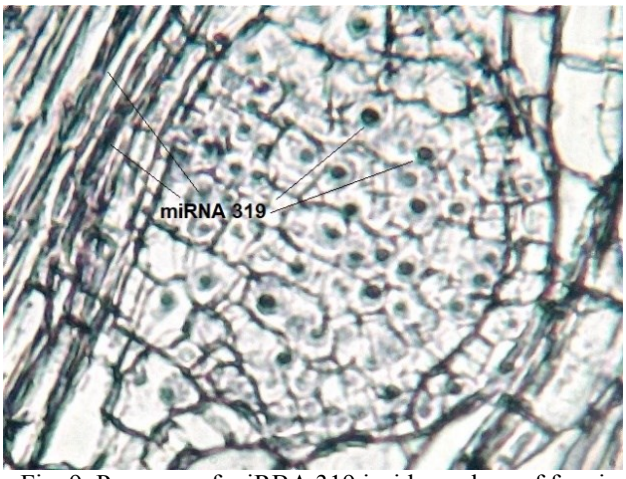

Fig. 9. Presence of miRBA 319 inside nucleus of fungi cells in tomato vesicle.

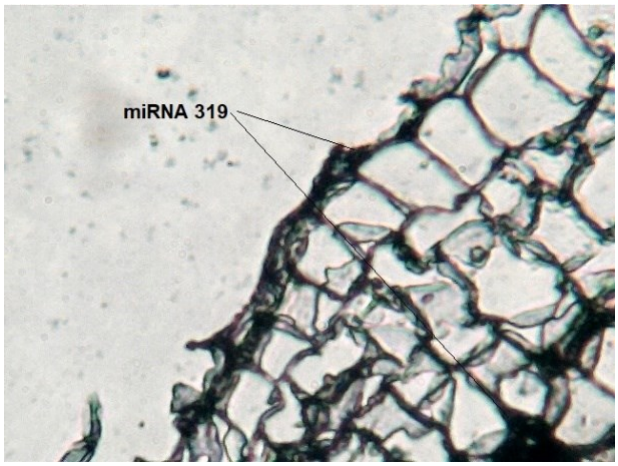

Fig. 10. Presence of miRNA 319 in external and internal hyphae.

The dark-blue color indicates presence of miRNA 319 in tomato cells but also in fungi cells of vesicles and hyphae. Especially inside nucleus of fungi cells of internal vesicles. No blue-dark color appears inside cells of *Alium cepa* skin cells used as negative control.

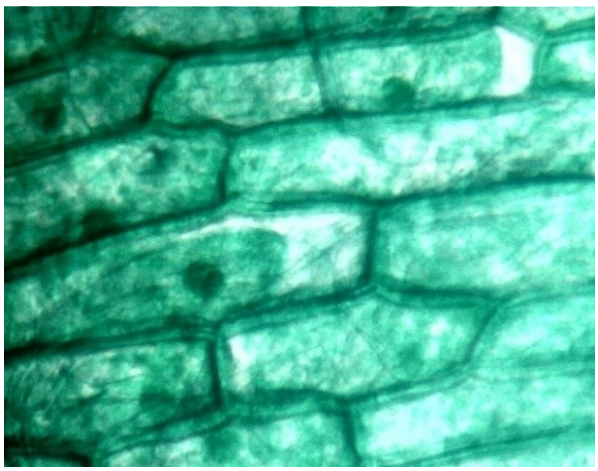
Fig. 11. Negative control on onion skin (Alium cepa).

We can confirm the presence of miRNA 319, not only in tomato roots, but also very dense in vesicles, arbuscules and intramatrical hyphae of tomato roots.

IV. Discussion

Use of mycorrhizae tomatoes, offers some yields in size of the plants and quality of the fruits, equal or superior to the best traditional crops (28) Authors have already shown in a previous article (4), the existence of early warning signs for the whole tomato crop. The ability of these signals to give an efficient response against environmental infections has been amply demonstrated in different crops. In the case of tomatoes, sentinel paper is more evident in HAC plants (high content in anthocyanins). However, the nature of these signals is not clear at all. miRNA 319 is a good candidate to explain the physiological response of tomato plants (29,30). Nevertheless, the presence of the miRNA, in the symbiotic fungi cells in the roots of the tomato, this not explain, how they are transmitted from one plant to another. Molecular transport experiments should be necessary, with the radioactively labeled molecule (miRNA 319) to study the molecular flow from one plant to another through the hyphae of the mycorrhizae mycelium.

V. Conclusions

Symbiotic relationship between tomato roots and mycorrhizae needs physical proximity between plants and a certain depth of roots to be establish. Sentinel effect of HAC plants is clearly beneficious for crop production. Presence of miRNA 319 related to the control of growth (production of abscisic acid) and the defense against diseases is well known. Presence in a concentrated way in arbuscules, vesicles and hyphae, makes this molecule a good candidate for the transmission of information through the mycelial web.

Acknowledgment

We would like to acknowledge to Bidasoa County Hospital, Histopathology Area, their kind help, in staining of plant tissues and to Rotary Club of San Sebastian – Donostia, for their grant to these research. And of course, to our colleges and friends from Basque Culinary Center, specially to Miguel Angel Lopez and Catherine Montes.

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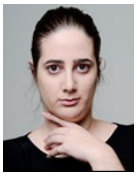

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