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Study of synthetic and natural substances able to activate defenses against pathogens in tomato plants, *Solanum lycopersicum L.*

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ABSTRACT

Solanum lycopersicum L. is a plant whose cultivation in the country, both open and greenhouse is subject to the use of a lot of chemicals to make it economically viable, since it is affected by a variety of pests and adverse environmental conditions. Tomato consumption is widespread in our country because of its taste, low calories and antioxidant properties. In order to fight plant diseases a variety of substances have been applied, ancestrally using plant extracts has been replaced by the use of chemicals in large areas of crops.

For the present work, several genes were chosen, which act as transcription factors showing higher expression levels in tomato leaves, genes encoding pathogenesis related proteins or PR and MAPK regulators. Gene activation of PR1a, PR1b, SlWRKY 8, SlWRKY 23, SlWRKY 39, MAPK 3 and MAPK 6 was assessed 24 and 48 hours after the application of substances of plant origin, electrolytic chemical and a commercial fungicide. The tomato plants were grown under controlled laboratory conditions. Once the treatments were applied, RNA extraction processes were carried out prior freezing of plant material in liquid nitrogen. Real Time PCR and relative quantification will be used to analyze the expression of the cited genes in tomato plants after the application of the treatments; the results were statistically analyzed with ANOVA and Tukey's HSD test to discriminate the effectiveness thereof.

The present work shows the induction of plant defenses by means of plant extracts which are widespread and easy to find. Aqueous extracts of chili pepper “Rocoto”, wild rue and ethanolic extracts of clove powder were spray tested on tomato plants, inducing the expression of different defense genes (PRs and regulatory proteins). The strongest induction was obtained with wild rue and clove. Infiltration tests in tomato and tobacco plants were tested, showing necrotic symptoms associated with the hypersensitive response, thus confirming the priming capacity of the extracts.

HPLC for salicylic acid (SA), and callose analysis were done in tomato leaves to verify the response. The results obtained suggest that natural antimicrobial extracts can be used to induce plant defenses and protect valuable crops. At the same time these low-cost extracts do not pose a threat to the environment or the farmer and can help reduce the farming costs, especially in developing countries.

Keywords: tomato, PR1a, PR1b, WRKY, MAPK, transcription factors, RT-PCR.

RIASSUNTO

Solanum lycopersicum L. è una pianta la cui coltivazione è diffusa in Ecuador e nel mondo, all'aperto e in serra. Il consumo di pomodoro è molto diffuso grazie al suo sapore, al basso contenuto in calorie e alle sue proprietà antiossidanti. La coltivazione del pomodoro è soggetta all'uso di molte sostanze chimiche per renderla economicamente fattibile, poiché è influenzata da una varietà di parassiti e condizioni ambientali avverse. Al fine di combattere le malattie delle piante sono state applicate molteplici sostanze: negli ultimi decenni l'utilizzo ancestrale di estratti di piante è stato sostituito con l'uso di sostanze chimiche nelle grandi aree di colture.

Il presente lavoro mostra l'induzione delle difese delle piante per mezzo di estratti di piante, che sono diffuse e facili da trovare. Estratti acquosi di peperoncino "Rocoto", ruta selvatica ed estratti etanolici di polvere di chiodi di garofano sono stati testati su piante di pomodoro valutando l'espressione di diversi geni di difesa (PR e proteine regolatrici). Diversi geni sono stati scelti per il presente lavoro che codificano per fattori di trascrizione della famiglia WRKY (che mostrano livelli di espressione più elevati in foglie di pomodoro), geni che codificano le proteine legate alla patogenesi (PR) o regolatori di PR (MAPK). L'attivazione dei geni PR1, PR2, PR3, SlWRKY 8, SlWRKY 23, SlWRKY 39, MAPK 3 e MAPK 6 è stata valutata 24 e 48 ore dopo l'applicazione di sostanze di origine vegetale. PCR quantitativa e quantificazione relativa sono state utilizzate per analizzare l'espressione dei geni citati in piante di pomodoro dopo i trattamenti; i risultati sono stati analizzati statisticamente con ANOVA e Tukey HSD per valutare l'efficacia.

L'induzione più forte è stata ottenuta con gli estratti di ruta selvatica e chiodi di garofano. Test di infiltrazione in piante di pomodoro e tabacco hanno evidenziato sintomi necrotici associati alla risposta ipersensibile, confermando la capacità di priming degli estratti.

Analisi HPLC dei livelli di acido salicilico (SA) e analisi della deposizione di callosio sono state fatte in pomodoro per verificare la risposta indotta. I risultati ottenuti indicano che gli estratti antimicrobici naturali possono essere utilizzati per indurre le difese vegetali e proteggere le colture pregiate. Allo stesso tempo, questi estratti a basso costo non rappresentano una minaccia per l'ambiente o l'agricoltore e possono aiutare a ridurre i costi di coltivazione, in particolare nei paesi in via di sviluppo.

Parole chiave: pomodoro, PR1A, PR1b, WRKY, MAPK, fattori di trascrizione, RT-PCR.

CHAPTER 1

INTRODUCTION

CHAPTER 1

1. INTRODUCTION

1.1 IMPORTANCE OF FOOD PRODUCTION

Around the world during the past century, there have been many events that forced humanity through periods of more or less prolonged hunger. Among these are counted wars and diseases that devastated many countries, and also pests that decimated agricultural production.

Famines have been common throughout the history of mankind. There was famine in ancient Egypt, and the book of Genesis in the Bible speaks of seven years of fat cows related to good harvests, followed by seven lean years, related to drought and poor harvests during that number of years, causing widespread famine.

In Europe there was a long period of famine in the early seventeenth century. At that time the cities were growing rapidly and, in years of poor harvests, town inhabitants had better paid jobs and could buy food while farmers had to sell what little they had cultivated to buy seeds needed to plant again.

A global view since 1850 to 2000s, indicate that from 1860 to 1890 about forty two million people died, and from 1920 to 1960 about seventy one million people died by cause of famines. In most recent times famous famines are the ones in Ethiopia (1983-1985). Among the worst famines in history are the ones from twentieth century linked to war and ideology more than crop failure or climate. (Ó Gráda, 2007) Poverty is one of several causes of hunger, but also harmful economic systems, conflicts, increasing population, and climate change. (Hunger, 2016)

We need a sustainable agricultural production to feed current and future habitants in our planet. In the coming years we will need enough food for a growing population and sources of inclusive jobs for those who are related to this activity. (Institute, 2013-2014)

Before the development of agriculture and grazing, 15,000 to 10,000 years ago, the livelihoods worldwide were primarily hunting animals, fishing and gathering wild plants. Although the diet of societies differed in a wide range of foods,

including those who only ate meat, had a vegetarian diet, to those who had a balance in their nutrition. (Biesalski & al., 2002)

Archaeologists have contributed to understand what kind of food the early civilizations used to have when they analyzed by radiocarbon dating the objects found in prospections of early settlements. All the traces leaved by early civilizations are customs testimonials when they lived in organized human groups, for example fossils, parts of plants and animals, supplies used for their daily work, paintings on rocks.

The collection of food products depended solely on the season of the year availability. The origin of agriculture is estimated in the Neolithic 8000 to 10000 years ago when people around the world began to abandon foraging lifestyle. (Wadley & Martin, 1993)

The first sedentary societies favored the development of permanent settlements, as well as new techniques and materials for cooking and storing food. Neolithic stone objects were polished, with new forms better in finish and effectiveness, but more important than the polishing was the appearance of pottery about 8000 years ago B.C., a fact undoubtedly influenced by the need to store surplus crops and cook food, which represented a marked improvement in the nutritional regimen. From this period are also basketry techniques with fine threads, fabrics and clothing with certain vegetable fibers or sheep's wool.

Theories state that the change from foraging to agriculture was gradually, some stimulus, stress, or environmental fact led tribes settle in one place and started to grow some of the food while part of it needed to be collected, until they switch to a lifestyle that depended completely on agriculture. (Levetin & McMahon, 2008)

Evidence of the beginning of agriculture has been found all over the planet, in Europe the Fertile Crescent (Iran, Turkey, and Israel), Yangtze and Yellow River Basins, Eastern North America, Mesoamerica, and South America Highlands.

Domesticated plants differ from the original ones in many aspects, their traits have been chosen to suit the population needs, leading to the loss of some qualities that gave them defense in the wildlife. Both domesticated plants and animals are genetically different from their wild relatives because they have been shaped by artificial selection once man began planting. (Levetin & McMahon, 2008)

Forms of exchange between neighboring human groups then appeared, seeds and tubers where spread to surrounding regions. Principle crops in the world started to develop, in Europe wheat and barley, in Africa sorghum and millet, rice in the Far East, corn in Mesoamerica, potato and other root crops in South America.

With the domestication of animals, they were used to help in farming activities, increasing farmers ability to cultivate larger fields. During the 1800s mechanized farm equipment started to appear, as the number of farms diminished, their size were larger. Land availability for farming became a limiting factor. (Evans, 2001)

In order to increase crop yield, fertilizers and pesticides were used in the early 1900s, tractors and other mechanized machines were developed over the next several decades. Around 1960 industrial methods in agriculture were established, also the use of agrochemicals and mechanized methods of farming.

The Green Revolution reached in developing countries during 1960s, the productivity of global agriculture was increasing as a result of new plant varieties (high-yield-crops), improved fertilizers and agrochemicals, irrigation and crop management techniques. It was an international effort to eliminate hunger by improving crop performance.

Norman Borlaug is considered the father of the Green Revolution, he eventually won the Nobel Peace Prize in 1970 for developing high-yield crops to prevent starvation in developing countries. The productivity of global agriculture could be increased drastically at the expense of the environment and society when large amounts of fertilizers and pesticides are used. In contrast, in many developing countries insufficient funds are available to provide fertilizers, pesticides and fuel necessary to achieve full potential of best cultivars. The staff of the Norman Borlaug Institute for Plant Science Research are committed to develope low input/low environmental impact and high yield/high quality cultivar strains. These cultivars will satisfy the need for efficient sustainable agricultural production in both developing and developed countries. (Prakash, 2011)

Industrial agriculture nowadays depend on synthetic fertilizers and pesticides, large amounts of irrigation water, major transportation systems, factory-style practices for raising livestock, and machine technology, thus creating risks for future food production.

The industrial farming model takes into account some aspects: adapting to new technologies, finding the best and appropriate technology, develop of standardized systems, learn from partners and extend the scope of business, seek management strategies, reduce costs, gain economy size, utilize automation, focus on product quality, recognize and emphasize buyer expectations in their choice of product and production practices, and develop closed loop systems that utilize all resources, including waste, as efficiently as possible. (Gray & Boehlje, 2007)

Biodiversity is the foundation of agriculture. Its maintenance is essential for food production and other agricultural products and benefits provided to humanity, including food security, nutrition and livelihood. Agriculture promotes biodiversity while it is reinforced. Sustainable agriculture uses water, land and nutrients with effectiveness while producing durable economic and social benefits.

However, the Earth's biodiversity is being lost at an alarming rate, endangering the ecosystem maintenance and the ability of agriculture to adapt to changing conditions. The conservation and sustainable use of biodiversity is essential to the future of agriculture and humanity. The Convention on Biological Diversity (CBD), with country members almost worldwide, provides a comprehensive framework for collective action among countries and citizens of the world to stop biodiversity destruction, promote sustainable use of the components of biological diversity and fair and equitable sharing of the benefits arising out of the utilization of genetic resources, basic to survival. (CBD, n.d.)

From the 27,000 species of higher plants, about 7,000 are used in agriculture. However, nowadays, only 30 crops provide approximately 90% of food energy needs for world population; wheat, rice and corn themselves providing about half of food energy consumed worldwide. Climate is the most important environmental factor affecting production and now is also influenced by agriculture. Approximately 24% of earth's surface is covered by cropping systems.

Agriculture accounts for 44% of anthropogenic methane emissions and 70% of nitrous oxide gas, mainly since the conversion of new land for agriculture and use of nitrogen fertilizers. (Biológica, 2008)

If we are looking for sustainable agriculture we must consider that maximum performance is not the main objective, but achieving long term stabilization.

Conservation of energy and resources, environmental quality, public health and equitable socio-economic development, crop species, rotations, spacing in rows, fertilization, pests control and harvesting should be taken into consideration. (Altieri M. A., 1999)

Barriers inhibiting its widespread adoption should be reduced, especially in developing countries where farmer participation has become essential for sustainable agriculture and also in developed countries an agro-ecological approach should support rural communities. One of the causes of crops diversity decrease is monoculture where plant species are grown in large areas with minimum or no rotation. (Frison, 2016) Around the world agricultural practices have been oriented to the cultivation of crops in the same space at the same time, known as polyculture or intercropping aming for an effective use of resources. (Lithourgidis & al., 2011)

Most of basic crop production in Latin American tropics comes from a polyculture system: more than 40% of cassava, and 80% of beans of those regions are grown combined together or with other crops. (Francis, 1976) The prevalence of these systems suggests that farmers are aware of the benefits thereof but they need crop varieties and management practices to improve the benefits of the existing systems. An important dilemma for development is how to transfer and adapt biotechnology to political, economic and social conditions prevailing in developing countries.

At present we observe a transition from traditional agriculture to agroecology in order to rescue the knowledge and practices of ancient agriculture production systems. Biological inputs and processes are required to restore soil productivity and develop both its fertility and productive stability, along with plants or species capable of developing defenses against a pest or disease.

1.2 CURRENT WORLD SITUATION

Global situation for food supply is a constant concern, population projections over the next 50 years are two billion additional people. (Nations U. , 2015) Taking into account that developed countries have the technology necessary to optimize land, water, and soil management, while developing countries may struggle to feed themselves and lower the quantity of imported goods.

“In recent years the growth rates of world agricultural production and crop yields have slowed. This has raised fears that the world may not be able to grow enough food and other commodities to ensure that future populations are adequately fed”. (NATIONS, 2002)

Although Latin America and the Caribbean had lowered the percentage of undernourished people according to the Millennium Development Goals and the World Food Summit goal, it is worrying the fields abandonment in search of a better economic situation. Monocultures have displaced traditional diversified small producers in Latin America where 70% of food production come from familiar agricultural systems. (Altieri M. A., 1999)

According to information provided by the FAO, the top five crops produced in descendent order are: sugar cane, maize, rice, wheat and potatoes. The majority of food consumed by each country is produced locally, another part can be imported to meet demand, values of international flows have increased around fivefold over the past 50 years, reflecting global trends in the overall trade volume. (Nations F. a., 2015)

Within the top 20 countries list for food production are: Brazil for sugar cane, Ethiopia cereal production, United States of America for maize, China for rice, wheat and potatoes. While among the top 10 commodities imported are: wheat, maize, soybeans, oil palm, rice, sugar, barley, and water. Top 10 commodities exported are: wheat, soybeans, barley, sugar and rice. (Nations F. a., Countries by commodity, 2013)

Tomatoes production in the world is led by China, India and the United States of America, while in Latin America, Brazil has the higuer one. (Nations F. a., Countries by commodity, 2013)

1.3 CURRENT ECUADOR SITUATION

In Ecuador land use in rural areas for permanent and temporary crops reaches 12’201.254 hectares, according to the Surface Survey and Continue Agricultural Production 2014. (Censos, 2014) Permanent crops represent 11.61 % while

transitory crops 7.18 %. Tomato production is within transitory crops, as can be seen in Table 1.

Table 1. Surface by category of land use in Ecuador. Source: ESPAC 2014.

NATIONAL TOTAL	12'201.254 hectares	100,00 %
PERMANENT CROPS	1.417.104	11.61 %
TRANSITORY CROPS	876.498	7.18 %
REST	93.574	0.77 %
CULTIVATED PASTURES	2.259.447	18.52 %
NATURAL PASTURES	828.333	6.79 %
PARAMOS	499.258	4.09 %
MOUNTAINS AND FORESTS	5.578.859	47.20 %
OTHER USES	468.160	3.84 %

Permanent crops represent 26.33 % of the agricultural labor area; principal crops are sugar cane, banana, and african palm. Transitory crops represent 16.29 % of the agricultural labor area; principal crops are corn, rice and potato. Loss of monocultures area (hectares) was about four times higher than in intercropping. (Censos, 2014) Land use change in the last 20 years in Ecuador: reduction of 4% in agricultural area, 12% in forest, 11% in other land. Figure 1. (Nations F. a., FAOSTAT, 2016)

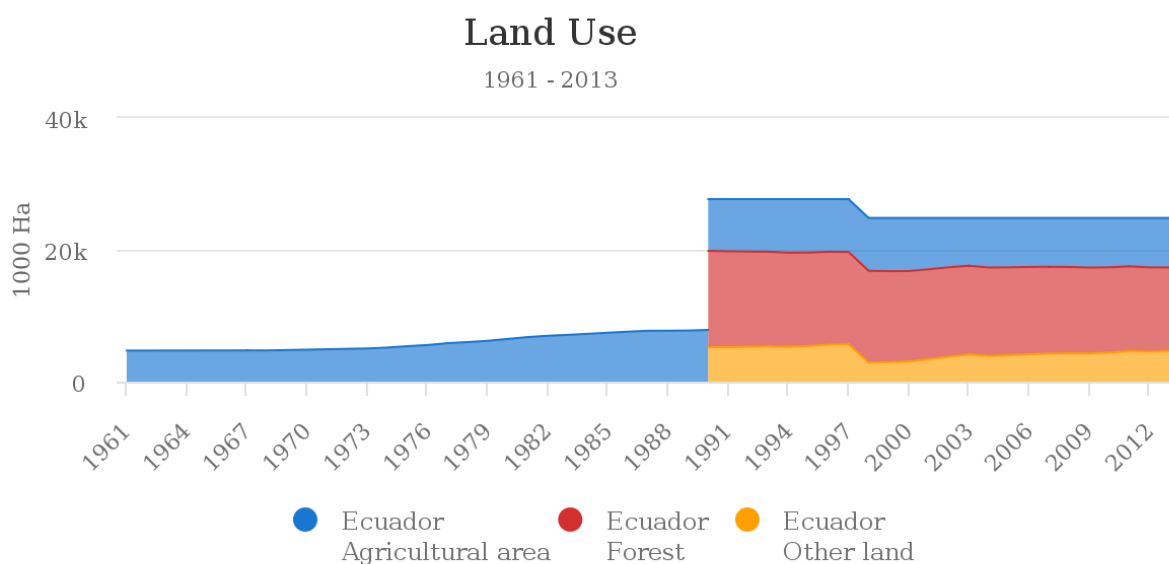

Figure 1. Land use in Ecuador. Source: FAOSTAT.

Agricultural area use has changed in the last 20 years in Ecuador: arable land reduced by 27%, permanent crops increased by 8%, and permanent meadows and pastures reduced by 1%. Figure 2. (Nations F. a., FAOSTAT, 2016)

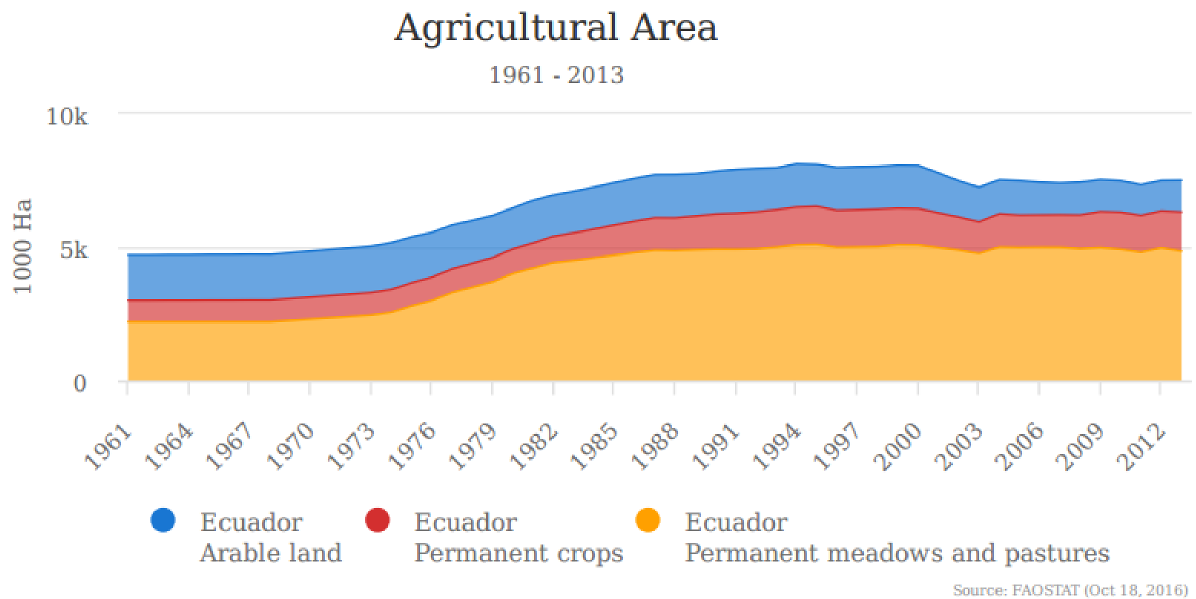

Figure 2. Agricultural area in Ecuador. Source: FAOSTAT.

According to data provided by the National Institute of Statistics and Census in Ecuador, (Instituto Nacional de Estadísticas y Censos INEC) to the National Information System of Agriculture, Livestock and Fishing (Sistema de Información Nacional de Agricultura, Ganadería, Acuacultura y Pesca SINAGAP), tomato production registered in Ecuador during the period 2006-2012 was 381,865 tons; and in 2012, 62,956 tons, corresponding to a planted area of 3,115 hectares and a harvested area of 3,077 hectares. (Ministerio de Agricultura, SINAGAP, 2013)

Harvested area for tomato production has decreased during the last 20 years about 50%, Figure 3, but yields have not changed significantly at around 211,352 Tons/Ha.

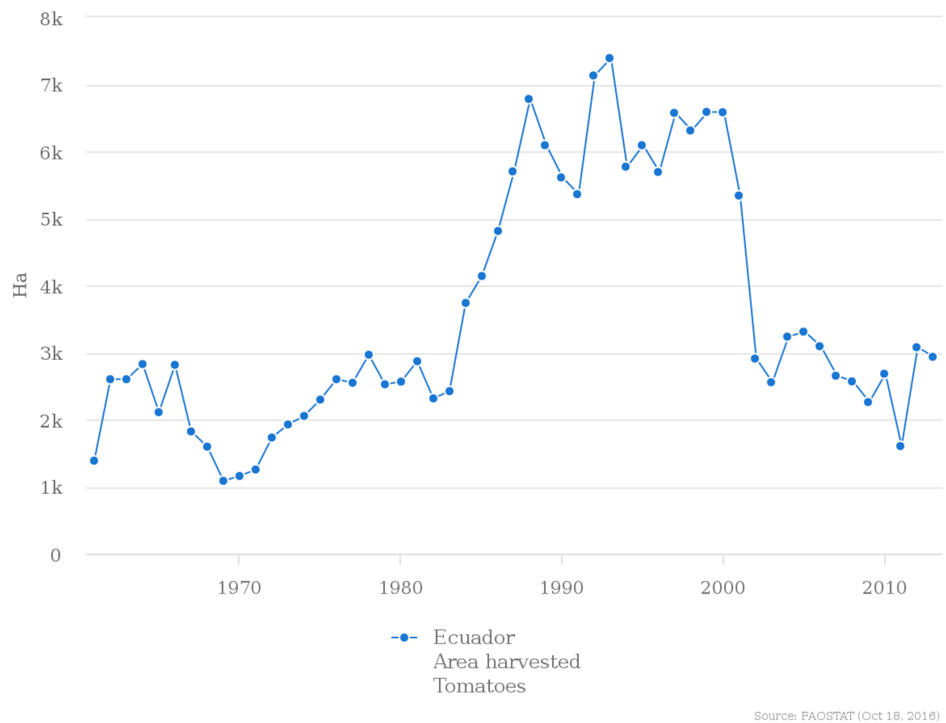

Figure 3. Tomato harvested area. Source: FAOSTAT.

One of the problems that concerns humanity is the production of CO_2 and N_2O gases both directly contributing to greenhouse effect turning the planet warmer. Carbon dioxide comes primary from the use of fossil fuel and by human induced impacts such as deforestation, agriculture, soil degradation. (Agency U. E., EPA , 2016)

In relation to this fact, food industry is responsible for 1/3 of the total gas produced in the world, N_2O comes from the production and use of nitrogen fertilizers, hence the need to seek sustainable alternatives to fix nitrogen in the soil naturally. (Nature, 2011)

Agricultural production, taking into account estimates from 2005, 2007 and 2008, released up to 12,000 megatonnes of carbon dioxide equivalent a year — up to 86% of all food-related anthropogenic greenhouse-gas emissions. Fertilizer manufacture releases up to 575 megatonnes, refrigeration emits 490 megatonnes. The whole food system released 9,800–16,900 megatonnes of carbon dioxide equivalent into the atmosphere in 2008, including indirect emissions from deforestation and land-use changes. (Vermeulen, Campbell, & Ingram, 2012)

In Ecuador CO_2 emissions have increased over the last twenty years by about 15%, in 2013 the emissions were 2,8 metric tons per capita. Figure 4. Carbon dioxide emissions have a tendency to increase, as shown by the analysis of the last 20 years. Figure 5. (Bank, 2013)

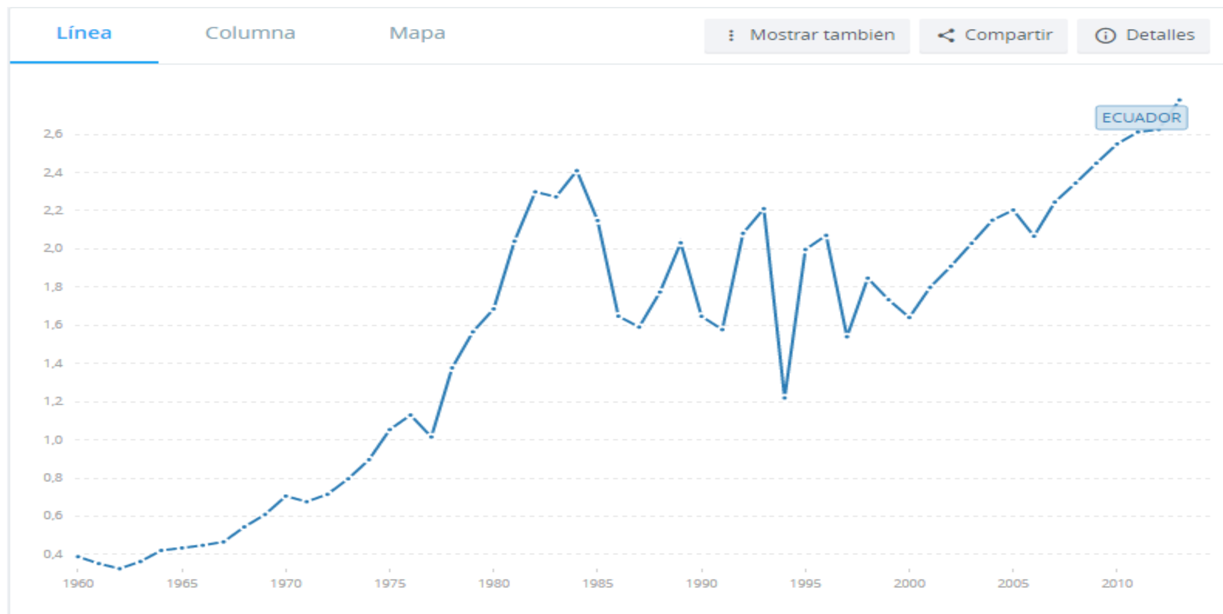

Figure 4. CO_2 emissions in Ecuador from 1960 to 2013. Source: WORLD BANK.

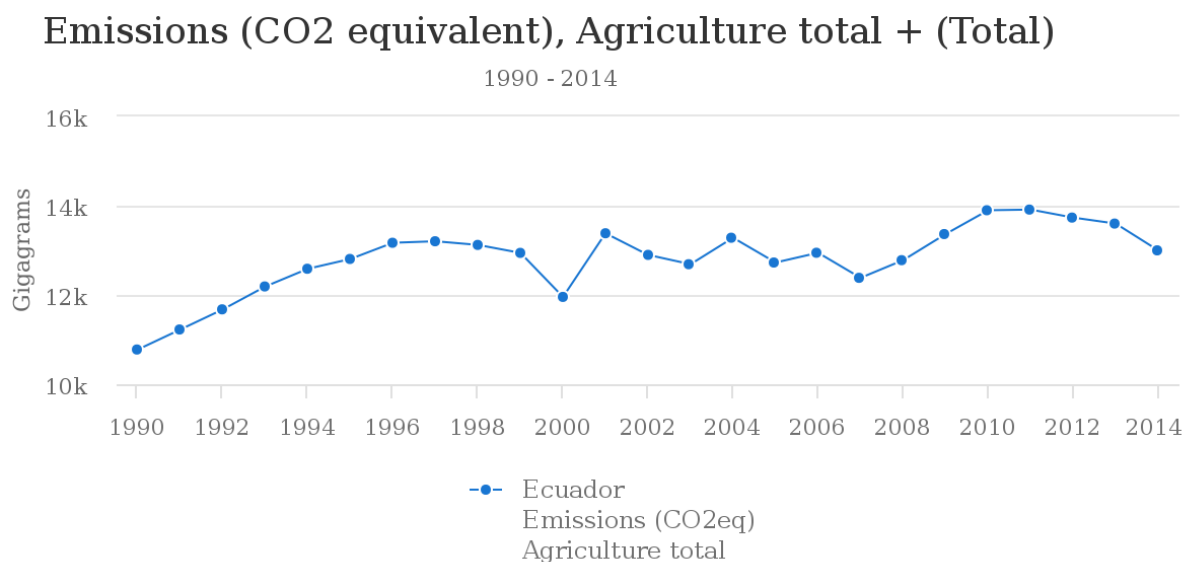

Figure 5. CO_2 Emissions Agricultural total. Source: FAOSTAT.

Analyzing the sources of greenhouse gas emissions caused by agriculture, three main sectors can be named: enteric fermentation that produces carbon dioxide

CO_2 and methane CH_4 during cattle digestion, followed by manure left on pasture, and synthetic fertilizers. Figure 6. (Nations F. a., Agriculture's greenhouse gas emissions on the rise, 2014)

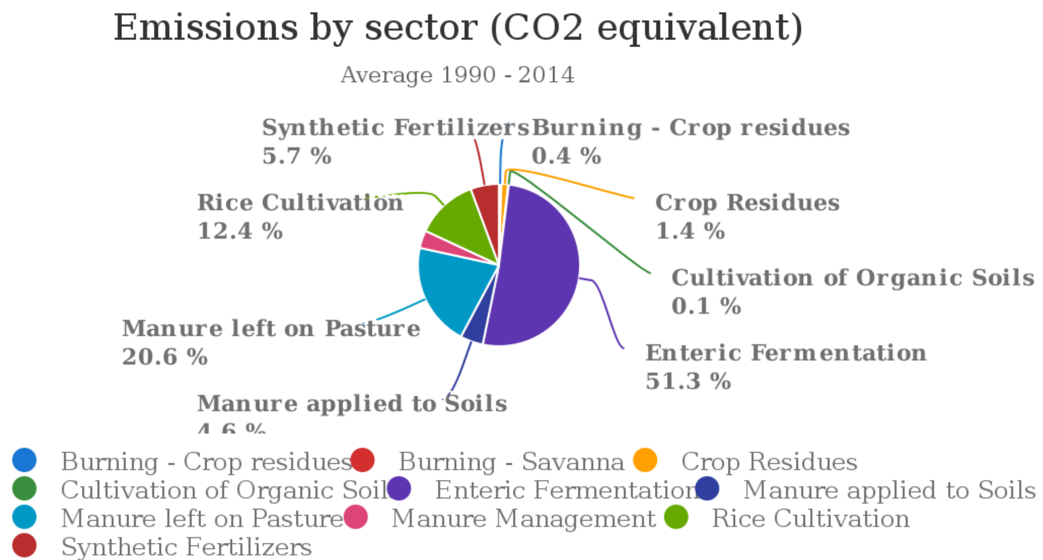

Figure 6. CO_2 Agricultural emissions by sector. Source: FAOSTAT.

The formation of concerned citizens that contribute to search solutions to agriculture challenges nowadays is an imperative. Become aware of new clean technologies that will achieve good yields without causing environmental damage using available resources and optimizing them is a duty towards future generations. Strategies should be sought to encourage agricultural production, improving market prices, valuing agro-ecological products free of agro-chemicals, boosting field production. But there are many social and cultural barriers that must be broken to achieve an understanding of how important is agricultural production for survival.

Formal agricultural education is not well received as it was in previous years. The list of university courses that are most in demand in Ecuador, quota allocation process by the National System of Equalization and Admission (Sistema Nacional de Nivelacion y Admision SNNA), establish for example that Agronomic Engineering is ranked nineteenth among twenty others; Technical and Technological Institutes for Agricultural and Agribusiness are ranked eighth and eleventh respectively.

According to a list of top careers, the most payed ones by sectors are: oil extraction and mining, finance, banks and insurance, private health professionals, none of them reflect the need to farm for food production. (Lideres, 2015)

Countries in general need to renew the interest for the rural family small production system that has been considered an economic loss as a result of labor demand and not favoring exportation, thus resources have been removed and redirected to export production systems.

1.3.1 CONVENTIONAL PRODUCTION

Conventional agriculture is based on two objectives: maximization of production and profits. To achieve these objectives, practices have been developed that do not consider the long term consequences and ecological dynamics of ecosystems, for example: the use of inputs, increased productivity, intensive farming, monoculture, irrigation, inorganic fertilizer application, chemical pest control, and crop genetic manipulation.

Intensive tillage tends to degrade soil quality in different ways. Organic material is reduced as a result of the absence of vegetation cover and soil is compacted by heavy machinery, this implies adding nutrients and using more machinery to break compaction. Tillage also increases significantly the intensive soil erosion produced by wind or water. Large scale monoculture facilitates machinery use minimizing manual labor, increasing chemical pest control and application of inorganic fertilizers, with consequent water pollution. All management practices used in conventional agriculture tend to favor short term high productivity, compromising crop productivity in the future. (Gliessman, 2002)

Along with large scale agriculture, monoculture has reduced the number of farms and farmers, especially in developing countries where mechanization and massive use of inputs are common, as proof of this the Food and Agriculture Organization of the United Nations FAO has stated that world's total arable land has decreases but production per hectare has remained.

1.3.1.1 AGROCHEMICAL USE

Modern agriculture has incorporated the use of agrochemicals in order to control pests and achieve high yields compared to traditional agriculture. Estimated worldwide annual sales of pesticides (herbicides, insecticides, fungicides, and others) in billions of dollars have increased in a meaningful way over the last fifty years. Figure 7. Despite greater awareness of the harm pesticides cause to the environment and human health, dependence on pesticides has not diminished. Furthermore simultaneous improvement of pesticides has made them more effective and application-specific, but also more toxic. (Agency E. E., 2016)

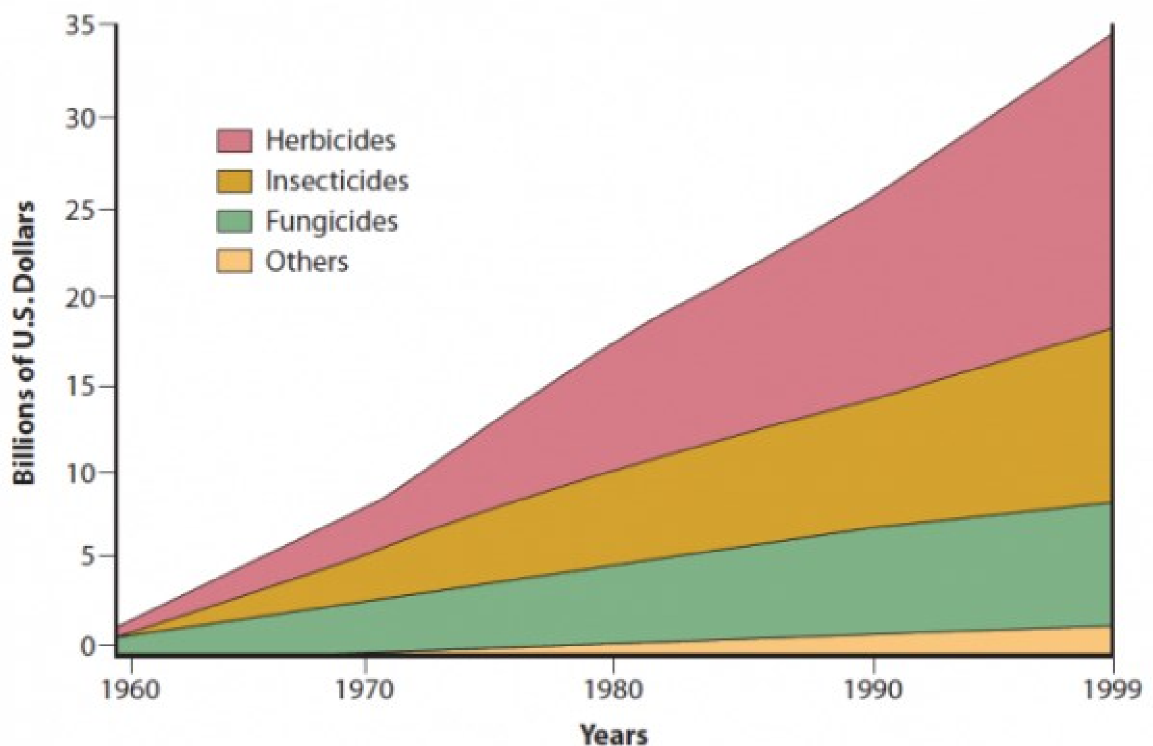

Figure 7. Billions of US Dollars spent in Agrochemicals. Source: FAOSTAT.

The amounts of agrochemical residues found in food must be safe and as low as possible for consumers. Maximum residue level (MRL) is the highest level of a pesticide residue that is legally tolerated in or on food or feed. To reduce pesticides use, the strategy aims to encourage low-input or pesticide free cultivation, in particular through raising user awareness, promoting the use of

codes of good practice and making financial means available for applied research and training. (Commission, New rules on pesticides residues in food, 2008)

The registration process takes into account the ingredients of the pesticide; the particular site or crop where it is going to be used; the amount, frequency, and timing of its use; storage and disposal practices. The evaluation process for a new pesticide includes the evaluation of human health risks, environmental risks, and risk assessments. (Agency U. E., About Pesticide Registration, 2016)

In Ecuador, considering year 2013, the total cultivated area was 1'320.988,67 hectares, 47% of which use some kind of chemical pesticide. In 12% of permanent crops hectares, pesticides are used without knowing their toxicity, this value goes down to 10 % in the case of transitory crops.

Selection for an agrochemical depends on many factors, the main criterion is its effectivity 44,3 %, as can be seen in Figure 8.

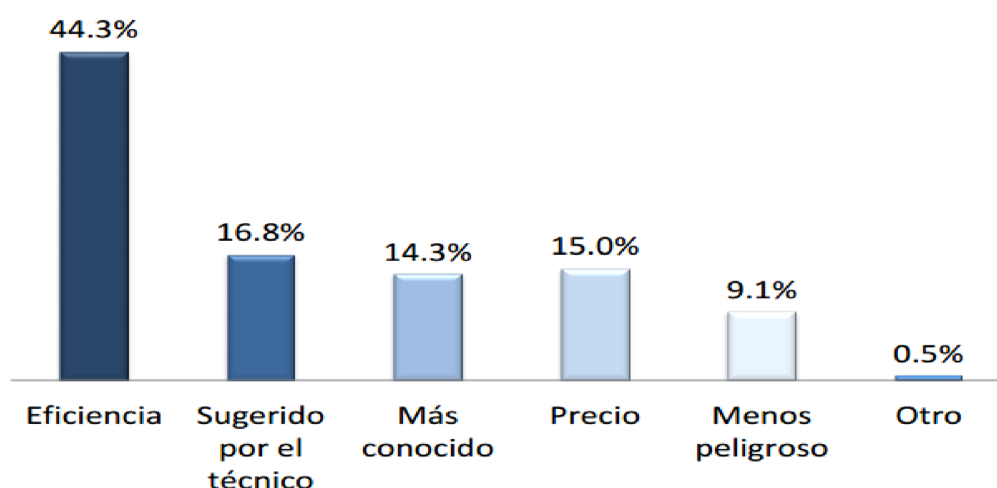

Figure 8. Surface where the main buying criterion applies (percentage hectares). From left to right: efficiency, suggested by the technician, most popular, price, less dangerous, other. Source: ECUADORENCIFRAS Uso de Plaguicidas en la Agricultura 2013.

Less than 40% of the farmers working in production areas are qualified or trained for the use of agrochemicals. Awareness in the storage of hazardous substances has been encouraged, the majority of agrochemicals are stored in warehouses and less than 5% remain inside households. (SINAGAP, 2016)

In 10% of transitory crop areas, pesticides are used without knowing their toxicity level. Figure 9.

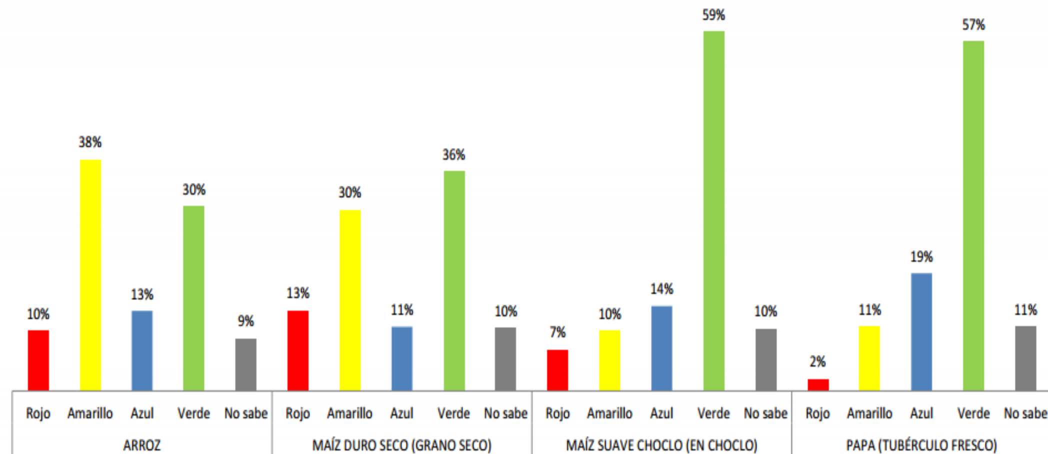

Figure 9. Hectares where pesticides are used according to label toxicity (percentage). Red is extremely toxic (category I), yellow is highly toxic (category II), blue is moderately toxic (category III) and green is slightly toxic (category IV). Source: INEC-2013.

From the ecological agriculture point of view, finding a balance between the ecosystem and production yield would be an ideal, avoiding the use of agrochemicals, hormones, which potentially damage the environment.

1.3.2 ANCESTRAL AND TRADITIONAL SYSTEM

Industrial agriculture is an exhausted model, the green revolution have failed, assuming that there would always be enough water and cheap energy, and that climate would not change. Also the dependence on agrochemicals, mechanization and irrigation has made it highly tied to fossil fuels. Industrial agriculture produce about 25% of greenhouse gases emissions. Nowadays there are about 1 billion hungry people on the planet; hunger is caused by poverty and inequality, not the lack of production. (SOCLA, 2012)

Conventional farming practices have mainly two objectives, maximize production and profits, without taking into consideration the long-term consequences. In order to achieve these goals, the main tasks of modern agriculture are: intensive

farming, monoculture, irrigation, application of inorganic fertilizers, chemical pest control and crops genetic manipulation. Intensive farming tends to destabilize soil quality, caused by organic matter decrease and compaction. The water used for irrigation is consumed faster than it is replaced, there is competition with the needs of urban areas and other species that depend on it. Agrochemicals can be harmful because they eliminate both pests and their natural enemies, pests resistance increases and also persistence causing negative environmental effects. Domestication of crops and natural selection have been strategies that mankind has used to choose the most appropriate crops; in recent years genetic engineering allows the creation of pest and agrochemical resistant plants whose long term consequences are not yet known, but some countries have banned their planting to defend the biodiversity. (Gliessman, 2002)

For agriculture to be sustainable and have the ability to renew itself, greater equity and benefits for everybody should be sought through cultivation practices, proper knowledge and ecological processes. Sustainable agriculture should cause minimal damage to the environment, preserve and rebuild soil fertility, using water allowing its natural recharge, rational use of resources, appraise and conserve biodiversity, and ensure equity in access to appropriate agricultural practices. (Gliessman, 2002)

“Sophisticated knowledge of the natural world is not confined to science. Human societies all across the globe have developed rich sets of experiences and explanations relating to the environments they live in. These ‘other knowledge systems’ are today often referred to as traditional ecological knowledge or indigenous or local knowledge. They encompass the sophisticated arrays of information, understandings and interpretations that guide human societies around the globe in their innumerable interactions with the natural milieu: in agriculture and animal husbandry; hunting, fishing and gathering; struggles against disease and injury; naming and explanation of natural phenomena; and strategies to cope with fluctuating environments”. (Nakashima, Prott, & Bridgewater, 2000)

According to the United Nations, the world population of indigenous people is approximately 370 million, this includes around 5000 different groups, living in over seventy countries. Indigenous people are defined as people whose social, cultural and economic condition distinguish them from others, being strikingly diverse in their culture and religion, and cherish their own living according to their traditions.

(Knowledge, 1997) They have maintained a close relation with the land, respecting the Earth and the life it supports, with the perception that humanity is one of many species. (Burger, 1990)

A long coexistence of farmers with nature has forced them to develop efficient solutions for a variety of problems through a process of trial and error. Traditional Andean farmers have an impressive ability to adapt to their natural environment, taking advantage of their potential and overcoming the limitations. Environmental quality includes the entire history of a product. Organic systems should strive to leave a small environmental footprint reducing environmental damage during the production process. Social quality of a product includes aspects such as fair payment to workers or producers of raw materials. The relationship between producer and consumer must also be considered. (Neckar-Verlag, 2001)

There is no doubt that humanity needs an alternative paradigm for agricultural development, one that fosters a biodiverse, resilient, sustainable and socially fair agriculture. The basis of these new systems are the variety of organic agriculture styles developed by at least 75% of the 1.5 million smallholders, indigenous and family farmers in 350 million small farms that represent no less than 50% of agricultural production for internal global consumption. (Group, 2013)

1.3.3 ECUADOR SITUATION REGARDING THE USE OF BIOINPUTS

There is a great interest for plans, programs and projects to design produce and promote the use of agricultural inputs (bio-products, organic fertilizers, biological control and other effective microorganisms) that replace agrochemicals which have been used during the last sixty years since the beginning of the green revolution.

Biopesticides, contrary to synthetic pesticides, come from living systems including plant and animal products with inhibitory effects; they can repel or destroy organisms that cause economic loss. One limitation of biopesticides is that they are not target specific, possessing a degree of effectiveness when they are applied in different edaphic conditions. At certain point it is considered that it will be better to blend bioproducts in order to complement the form of action.

Bio-inputs are beneficial to the environment and ecology, controlling agricultural pests, especially with the rising of organic products market. There are various types of biopesticides according to the target: insects, fungi, herbicides, nematicides, it is expected that from 2012 to 2017 the demand for biopesticides will grow at a rate of 16.1%. (Research, 2014)

The development of bio-inputs must overcome limitations such as public perception and lower shelf-life than synthetic agrochemicals considering the use of living organisms or processed plants, quality inadequacy, inconsistent field results, economic constraints and availability. A lot of work must be done to meet quality standards. (Kaushik, 2004)

Biopesticides currently represent only a small fraction of the world pesticide market. Various economic forecasting services estimated the world market for pesticides in 1995 at approximately $29 billion. Biopesticides are currently marketed for insect control, representing almost 4.5% of the world insecticide sales. (Menn & Hall, 1999)

Biopesticides research to develope better and stable formulas, preventing its oxidation and protected against UV light should be performed, hence they could intervene in organic farming in a more reliable way.

Some plant extracts have also been used to control diseases, for example leaf extracts of *Azadirachta indica* (neem), *Calotropis gigantean*, *Eucalyptus sp.*, *Parthenium hysterophorous* and *Pongamia pinnata* against fungal Fusarium infections. Leaf extracts were tested in various concentrations, showing different patterns of inhibition in the mycelium growth. (Joshi, 2006)

In Ecuador the Ministry of Agriculture, Livestock and Fisheries (MAGAP Ministerio de Agricultura, Ganadería, Acuacultura y Pesca) has regulations and technical standards to import agricultural inputs and supplies, also has designed rules for commercial production of agricultural inputs.

Reducing the environmental impact of integrated production systems requires returning to an agricultural production that would be clean and sustainable. In order to meet this objective, promoting sustainable agriculture encourage the use of biocontrol agents and bio fertilizers based on beneficial microorganisms, allowing the replacement of agrochemicals and therefore decreasing these products importation.

In recent years there have been several efforts to encourage agricultural population to use bio-products, initially at the level of communities where local production in small facilities were established. However, by vitue of the lack of confidence in the products, some laboratories have closed and the few remaining need to be supported to continue their work, for example Trichoderma bio-products in the province of Azuay, Paute village. (Ministerio de Agricultura)

1.3.4 AGROECOLOGY PERSPECTIVE

The practice of agroecology is old and it dates back to the origins of agriculture, although the contemporary use of this term reappears in the 70s. Many researchers have explored the indigenous agriculture, a modification of oldest agronomic forms. Agroecology rediscovery is an example of old technologies that are still valid and are being tested by science when studying what farmers had already learned to do, scientists have validated and explained this activities but not improved them.

Agroecology is an approach linked to the environment and is more socially sensitive, going beyond the agricultural production but based on ecological sustainability of the production system. Ecological criteria such as predator-prey relationships and crop competition with weeds are important.

Modernization has not reached poor farmers in Latin America, although it has increased agricultural productivity and total production, it has also brought significant environmental and social consequences in many regions. Modernization has not succeeded in improving small farmers agriculture, since it has relied on technologies (conventional agricultural practices) that displace nature and increase the gap between social and ecological processes. Industrially produced fertilizers substitute the relations between plants and nitrogen fixing bacteria, saturating agroecosystems instead of working with them. Pesticides and insecticides have displaced natural balance mechanisms exerted by predators and parasites, replacing therefore risk control methods that have an ecological basis. (Altieri M. A., 1999)

Researchers from agricultural sciences seek to understand the production mechanisms and propose innovations when necessary. Social science

researchers observe the production social organization that provides the key to labor and power relations within a given group. The current approach displays an integrated production system which combine biological, economic and social aspects to notice farmer and his family as a productive resources unity. (Eresue, M., Malpartida, & Poupon, 1990)

Many rural communities in Latin America have developed multiple cropping systems and rotations. Mutualistic association of crops with various possible mixtures of annual plants such as: short and large sized like maize and beans, high species where growth rate is different, early crops, crops of similar size with different growing seasons, crops of small size and rapid development with slow initial crop development, crops with differential susceptibility to environmental conditions, crops that complement nutritionally. Agroecological experimental evidence indicates the importance to maintain biodiversity mechanisms to stabilize pests. Low potential for crop pests can be expected with crops that exhibit the following: highly diverse mixtures of plants, intercropping and rotations; varietal crop mixtures of the same crop, discontinuity of monocultures, and periods without crops, small or scattered mosaic crop fields and uncultivated land to serve as a natural refuge for natural enemies. (Altieri M. , 1996)

To return to sustainable agriculture the following postulates must be met: maintain soil ecological health, be environmentally friendly, responsible water use, valuing and conserving biological diversity, use available resources within the agroecosystem, access to appropriate knowledge by technology developing and agricultural practices. (Gliessman, 2002)

CHAPTER 2

TOMATO – *Solanum lycopersicum* L.

CHAPTER 2

1. TOMATO - *Solanum lycopersicum L.*

2.1 TOMATO A DESCRIPTION OF THE PLANT

Tomato plant, *Solanum lycopersicum L.*, is a dicot shrubby plant of the *Solanaceae* family, native to western South America along the coast and high Andes from central Ecuador, through Peru, northern Chile, and in Galapagos Islands. It was introduced to Europe in the sixteenth century. Figure 10.

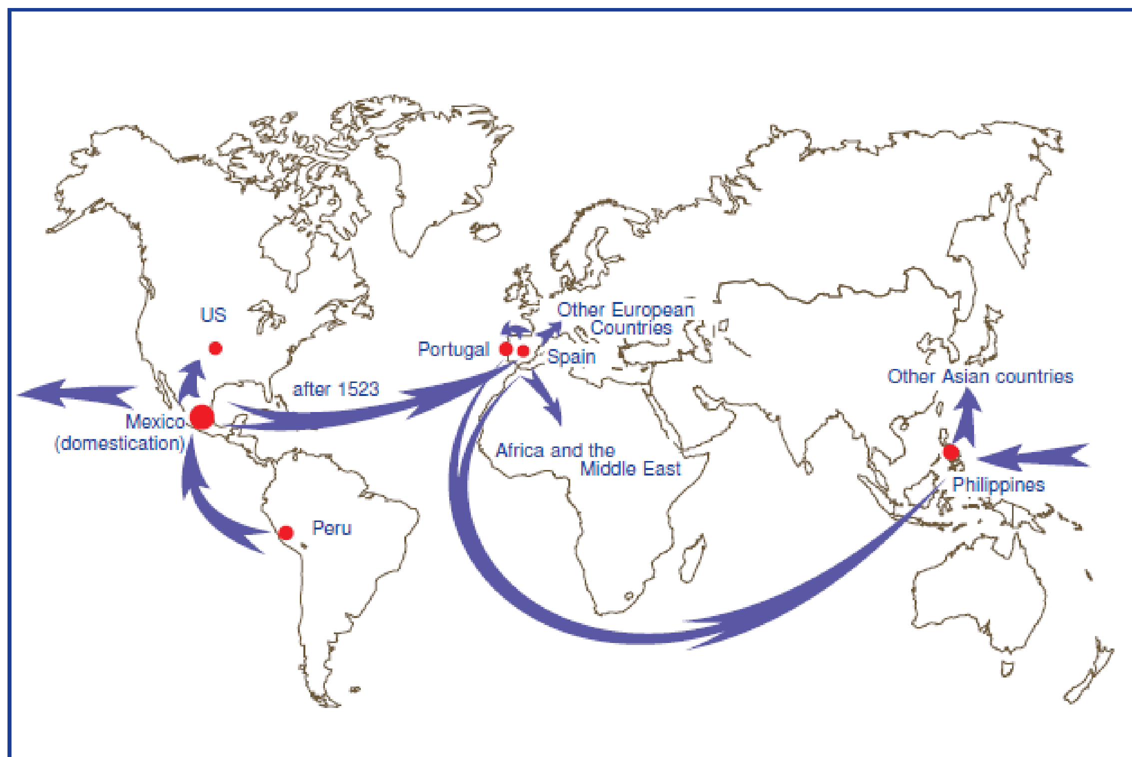

Figure 10. Map depicting the possible expansion of the tomato crop worldwide. Source: Tomato Diseases – Dominique Blancart – Academic Press 2012.

Taxonomic Lineage: cellular organisms; Eukaryota; Viridiplantae; Streptophyta; Streptophytina; Embryophyta; Tracheophyta; Euphyllophyta; Spermatophyta; Magnoliophyta; Mesangiospermae; eudicotyledons; Gunneridae; Pentapetalae; asterids; lamiids; Solanales; Solanaceae; Solanoideae; Solaneae; Solanum; Lycopersicon. (Center for Bioinformatics, 2016)

Tomato seed is flattened and has lenticular form with approximate dimensions of 3 x 2 x 1 mm. If they need to be stored for extended periods of time it is advisable 5.5% moisture. Quality seeds should have a germination percentage above 95%. Tomato root system consists of a main root, secondary roots and adventitious, generally it extends superficially over 1.5 m in diameter and reaches more than 0.5 m in depth; however, 70% of the roots are located within 0.20 m from the surface. (Perez, Hurtado, Aparicio, Argueta, & Larin, 2001)

While wild tomatoes can grow in many natural habitats like perennial herbaceous plants, cultivated tomatoes behave as annuals, semi-erect or erect depending on the variety, self-pollinating, bearing more than 1.5 meters tall. The plant cycle varies depending on environmental conditions and seed type, ranging from 3.5 months to 4 months. (Razdan & A.K., 2006)

The name “tomato” is derived from the Nahuatl language “tomatl” in Mexico corresponding to *Physalis philadelphica*; while *Lycopersicon esculentum* was called “jitomatl”. The early Italian name “pomi d’oro” (pomodoro) is still used in Italy. (Peralta, 2008)

Its leaves are compound and imparipinnate, 15-45 cm in length. Leaflets are 5-9 per leaf, ovate or oblong, 5-7 cm long, with toothed or lobed edge, acute apex and oblique base. Pseudostipuls are present, bisexual flowers in cymes of different branching patterns with 9-12 mm closed pedicels tops simple or bifid. Bracts are absent. Calyx deeply five-lobed. Yellow corolla is more than 2.5 cm in diameter. Red, pink or yellow, oblong or pear-shaped and depressed globose berries are more than 2 cm in diameter, hairless and plurilocular. It is a diploid plant with 2n = 24, among which are many single gene-mutants very important for selection purposes. (AgroEs, 2006)

The fruits can be classified into calibers and categories. Calibers take into account the transverse diameter of the fruits, the cherry type tomatoes have diameters smaller than 10 mm, and larger tomatoes can be classified in smaller than 70 mm diameter and greater than 70 mm diameter. The categories refer to the presence of major and minor defects in the fruit. Serious defects include decay, epidermis injury, necrotic areas, ripening or senescence. Mild defects include stains, hollow fruit, deformations, immaturity. The extra fitted to category presents no serious defects and may have one slight defect. Category I may have four serious defects

and 10 minor defects. Class II can have seven serious defects and 15 minor defects. (FAO, El Cultivo de Tomate con Buenas Practicas Agricolas en la Agricultura Urbana y Periurbana, 2013)

2.1.1 PHISIOLOGY OF THE PLANT

For proper growth tomato plants require temperatures between 10 ^{o}C and 43 ^{o}C, being the optimal temperature range around 26 ^{o}C to 32 ^{o}C, preferring high temperatures during the day and low at night. Plants often survive temperatures outside the growth range however it can disturb plant establishment producing frost damage and fruit sunscald. (Camejo & al., 2005) Temperature, light and humidity play a determinant role for pollen correct formation to pollinate flowers.

Tomato phenological stages start with seed germination at approximately 10 days since planted, the plant will need an active root development. Cotyledons development then starts and until day 20 the main stem can have up to 10 true unfolded leaves. Apical buds begin to display and at day 60 approximately flowers start to show and open. Fruit formation follows, tomatoes are green at the beginning until they reach the final and typical size, maturation then starts, most of the plant resources are being used for fruit development. Once the fruits have been harvested, plants start the senescence.

2.1.2 MOLECULAR MECHANISM OF PLANT DEFENSE

Unlike animals, plants are sessile organisms, for this reason they need proper and quick answers when changes occur in the surrounding environment. In the course of evolution, plants have developed mechanisms to defend themselves against biotic and abiotic factors, different kind of environmental signals can trigger enzymatic and molecular signaling cascades that can intercommunicate by different mechanisms. These signaling cascades can cause the formation of reactive oxygen species ROS, hormone signaling (ethylene ET, salicylic acid SA, jasmonic acid JA, abscisic acid ABA), which in turn induce the expression of genes that will complete the overall defense reaction. (Shanker & Venkateswarlu, 2011)

Among the Solanaceae, tomato is the most intensively researched both in genetic and genomic studies. (Barone, 2008) Plants have pre-existing and also induced defense mechanisms against pathogen attacks.

Pre-existing defenses can be structural and are represented by both physical (cell wall) and biochemical barriers such as toxic compounds or secondary metabolites with antimicrobial properties present in either active or inactive precursors that can be stored in organelles. (Hodson & Bryant, 2012)

In induced or active defense, host cells must be able to distinguish, by membrane receptors, between its own signals and those generated by a pathogen and known as elicitors. (Garcia, 2004)

Upon recognition different defenses are activated, such as hypersensitive reaction (HR) in which cells around the infection site become necrotic; and activation of pathogenesis related (PR) protein genes, a term coined by Antoniw et al. in 1980, both for biotic and abiotic stress. (Edreva, 2005) Through evolution, plants have selected one or more resistance genes called “R genes” that protect them from infection and play a key role in pathogen recognition. (Sanseverino, 2010) (Hammond-Kosack & Jones, 1997)

This kind of proteins do not accumulate exclusively in the infected leaf locally, but are induced systemically through the development of systemic acquired resistance (SAR). There are indications of an induced systemic resistance (ISR) which is phenotypically similar to SAR and can be induced by non-pathogenic microorganisms without the oxidative burst and the accumulation of PRs, and by means of molecules such as hydrogen peroxide and nitrogen monoxide. (Van Loon & al., The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins, 1999)

Induction of PR proteins in various plant tissues is one of the major biochemical and molecular events when plants are subjected to pathogen infections such as viruses, bacteria and fungi. (Perez & Hurtado, Guía del Cultivo de Tomate, 2001) Induction can be achieved by applying signals or chemical elicitors, such as salicylic acid, ethylene, jasmonic acid, systemin, which interact with plant receptors activating defence responses.

PR proteins are thought to play a fundamental role in systemic acquired resistance (SAR). (Van Loon, Induced resistance in plants and the role of pathogenesis-

related proteins, 1997) Originally, five major PRs groups were characterized in tobacco plants by biochemical and molecular techniques and also by means of electrophoretic mobility. They were named from PR-1 to PR-5 and each group consisted of several members with similar properties, either sharing amino acid sequences, serological relationships, and / or enzymatic and biological activity. The PR-1 group is the most abundant of all, which reaches 1 to 2% of the total leaf protein. (Edreva, 2005). It consists of low molecular weight proteins of unknown biochemical activity. "Currently PR-proteins were categorized into 17 families according to their properties and functions, including β-1,3-glucanases, chitinases, thaumatin-like proteins, peroxidases, ribosome-inactivating proteins, defenses, thionins, nonspecific lipid transfer proteins, oxalate oxidase, and oxalate-oxidase-like proteins". (Ebrahim, Usha, & Singh, 2011)

Table 2. Classification of pathogenesis related proteins. Source: Van Loon L.C.

Families	Type member	Properties
PR-1	Tobacco PR-1a	Antifungal
PR-2	Tobacco PR-2	β-1,3-glucanase
PR-3	Tobacco P, Q	Chitinase type I,II, IV,V,VI,VII
PR-4	Tobacco 'R'	Chitinase type I,II
PR-5	Tobacco S	Thaumatin- like
PR-6	Tomato Inhibitor I	Proteinase- inhibitor
PR-7	Tomato P69	Endoproteinase
PR-8	Cucumber chitinase	Chitinase type III
PR-9	Tobacco 'lignin forming peroxidase'	Peroxidase
PR-10	Parsley 'PR1'	Ribonuclease like
PR-11	Tobacco 'class V' chitinase	Chitinase, type I
PR-12	Radish Rs- AFP3	Defensin
PR-13	Arabidopsis THI2.1	Thionin
PR-14	Barley LTP4	Lipid- transfer protein
PR-15	Barley OxOa (germin)	Oxalate oxidase
PR-16	Barley OxOLP	Oxalate oxidase-like
PR-17	Tobacco PRp27	Unknown

Despite the good characterization of tobacco PR-1 gene family, there is little information about the mechanisms and gene regulation mode. In Arabidopsis two

PR-1 genes have been described, one of them is not induced by pathogen attack; the other is used as a molecular marker for SAR. (Tornero, 1997)

Acidic PR-1a protein was the first to be purified and characterized in *Nicotiana tabacum*, and is the typical member of PR-1 proteins. The structure and members of the PR-1 family in tomato include both acidic PR1-a1 and basic PR-1b1 isoforms. Tomato PR-1a isoform has demonstrated similarity to tobacco PR-1a isoform. (Rivas & Proaño, 2010)

Furthermore WRKY transcription factors are transcriptional regulators able to regulate gene expression induced by: pathogens, senescence, abscisic acid (ABA), gibberellic acid (GA), salicylic acid (SA). They play an important role, both in regulating plant growth and development, and in response to many kinds of biotic and abiotic stresses. WRKY transcription factors are named after a 60 amino acids region of WRKY domain, which has a conserved N-terminal sequence. They are able to bind W-box type (C / T) TGAC (C / T) DNA sequences, located in the promoter region of many genes. (Ciolkowski, Wanke, Birkengihl, & Somssich, 2008)

WRKY genes are usually activated by signaling through the mitogen activated protein kinase (MAPK) pathway or MAPKs cascades, and calcium dependent protein kinases. (Mingyu, 2012) 81 WRKY genes have been identified in *Solanum lycopersicum* SlWRKY, classified into three major groups, of which the second group is divided into five subgroups. Group I contains two WRKY domain, each containing a C2H2 zinc finger motif. Group II contains a single WRKY domain, including a C2H2 zinc finger motif, which can be divided, in turn, into five subgroups (II-a, b, c, d, and e, respectively). Group III contains a single domain, a C2HC zinc finger motif. (Huang, 2012)

2.2 TOMATO CULTIVARS IN ECUADOR

Around the world different tomato varieties are cultivated for direct consumption as well as for food industry. (Perez & Hurtado, Guía del Cultivo de Tomate, 2001) Its fruit is consumed worldwide both fresh and industrially processed.

The higher productions are found in Asia, followed by the Americas, Europe, Africa and Oceania. Figure 11.

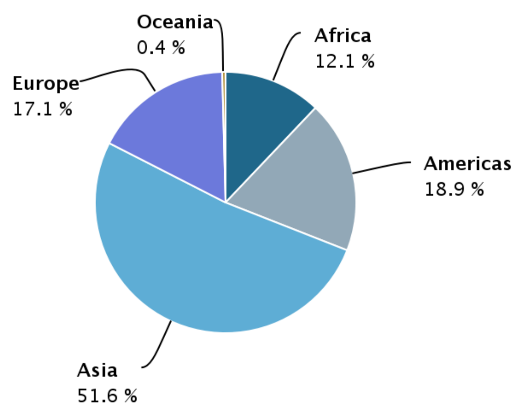

Figure 11. Production share by region Average 1990 – 2014. Source: FAOSTAT.

Tomato is one of many foods in the diet of ecuadorians, being low in calories and possessing antioxidant properties. In order to be commercially sustainable, cultivation depends on the use of agrochemicals. The most common causes for poor plant growth and crop destruction are plant pathogens, unfavorable weather, weeds, pests and insects.

According to data provided by the Food and Agriculture Organization of the United Nations FAO, Ecuador is among the countries showing lower production, <= 171.577 tons. Figure 12.

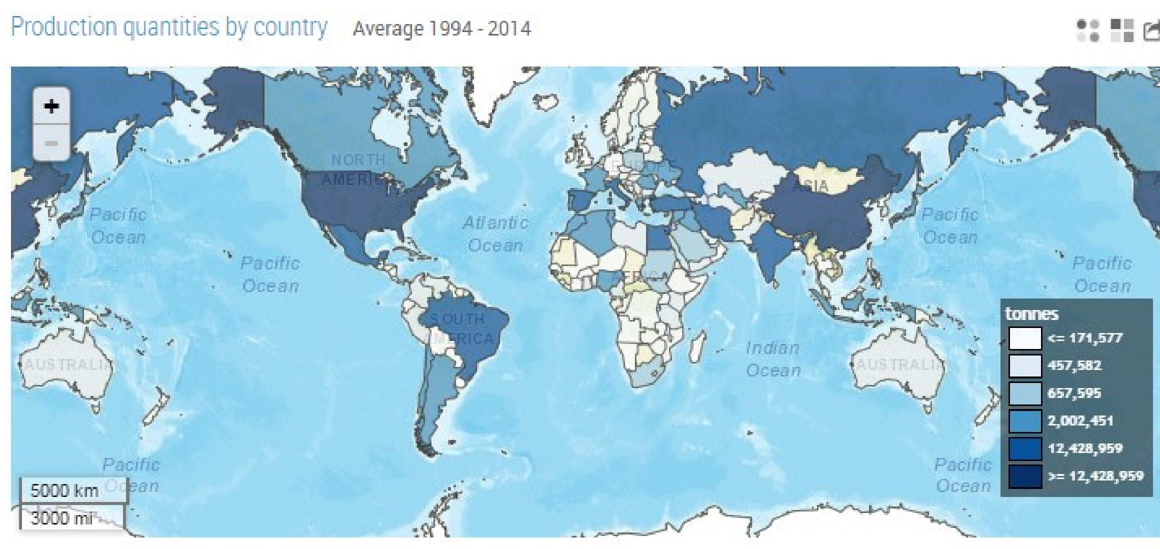

Figure 12. Production quantities by country Average 1990 - 2014. Source FAOSTAT.

Top five world tomato producers are China, United States of America, Oceania, Turkey and Egypt. In Latin America, Argentina, Chile and Brazil have important productions of tomato that is processed and exported to other countries, including Ecuador that needs to buy tomato paste to cover the industrial production of food with this ingredient. Figure 13.

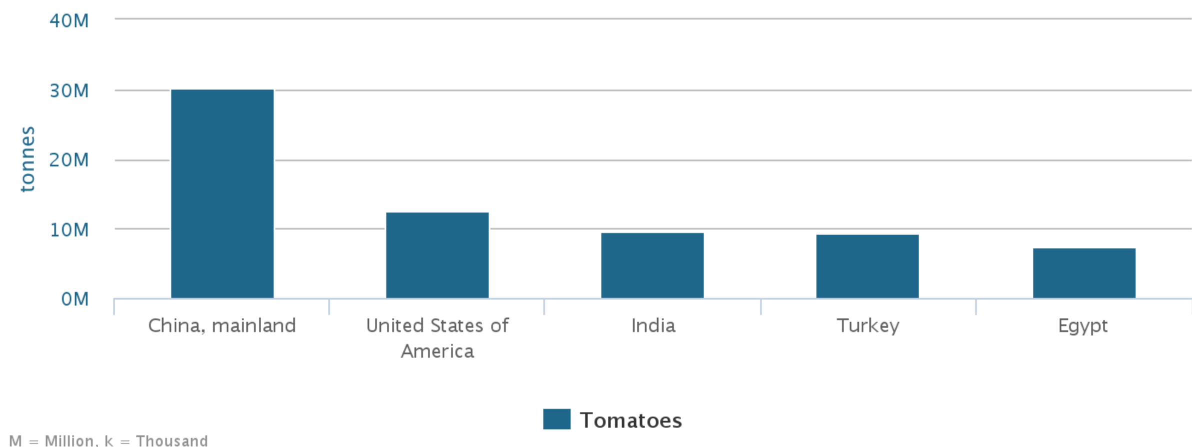

Figure 13. Production of top 5 producers Average 1990 - 2014. Source FAOSTAT.

The main and most profitable crops primarily produced in Ecuador are: sugar cane, bananas, oil palm fruit, rice paddy and maize. Figure 14. Other nonoil products that do not appear in the list of top but are exported worldwide are cocoa, coffee, shrimp, mango, passion fruit, heart of palm, pepper, broccoli, tuna, tilapia, toquilla straw, flowers, among others.

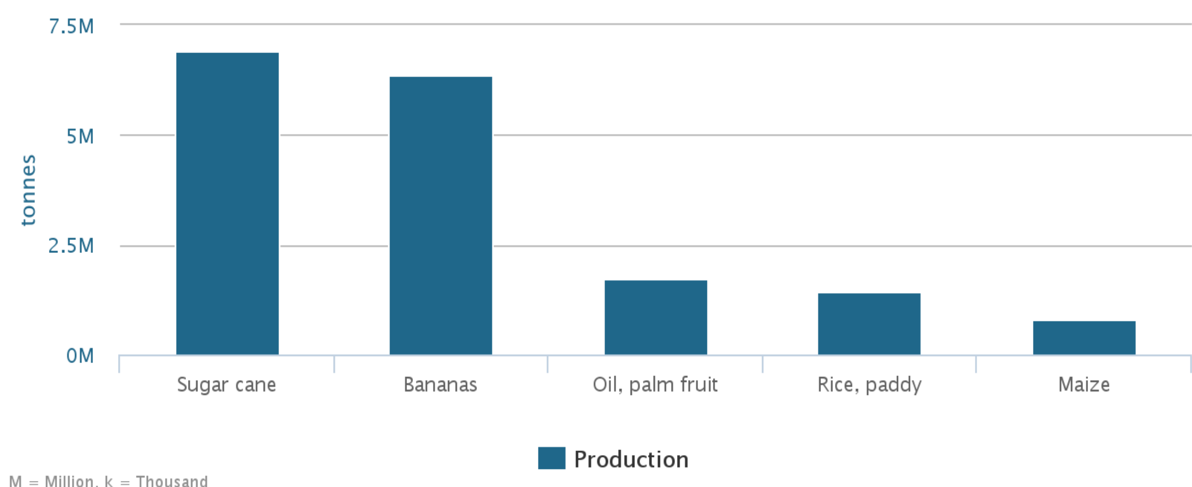

Figure 14. Most produced commodities in Ecuador Average 1990 - 2014. Source FAOSTAT.

Tomato production in 2013 has reached 64,736 tons in Ecuador, analysis of this figure shows clearly that there is not an upward trend in its production. Figure 15.

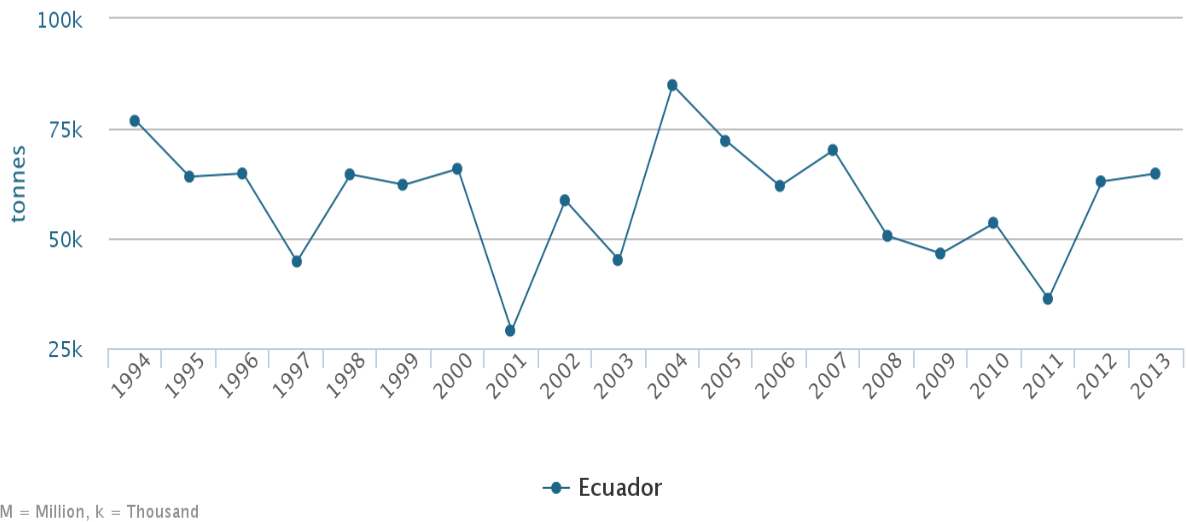

Figure 15. Tomato production in Ecuador. Source: FAOSTAT.

Yields in tomato production have increased during the last 13 years, maybe as a result of new agrochemicals use that enable better control of plant diseases. Figure 16.

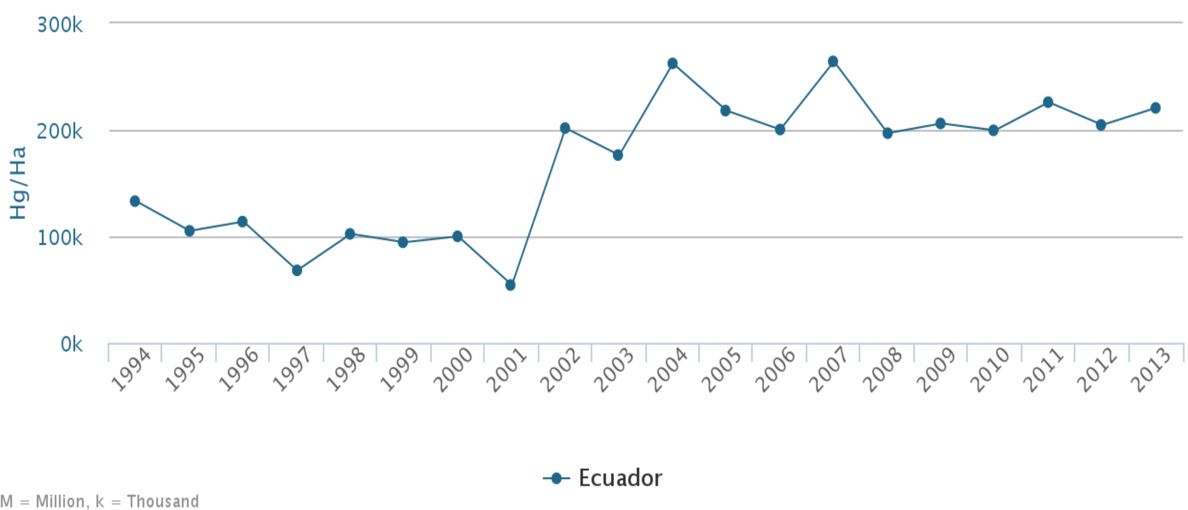

Figure 16. Yield of commodity in Ecuador 1990 - 2014. Source: FAOSTAT.

From this fact derives the interest to search and rescue alternative substances that do not represent potential danger for farmers, consumers and environment; and that, in turn, are feasible to use at large scale.

Transient crops in Ecuador have remained relatively constant from 2012 to 2015 in single crops, showing a slight decrease in 2011. The annual crops associated with other crops are presented in smaller quantities; in 2011 a peak of production appears.

Data provided for 2015 in Ecuador show that total planted area was 1,095,593 hectares, Figure 17, of which 71,057 hectares were lost corresponding to 6.5%, the harvested area was 1,023,974 hectares, Figure 18. Lost area by cause of pests is equivalent to 21,251 hectares corresponding to 1.9% of the total planted area, Figure 19; and lost area considering illness is 3,232 hectares corresponding to 0.3% of the total planted area, Figure 20.

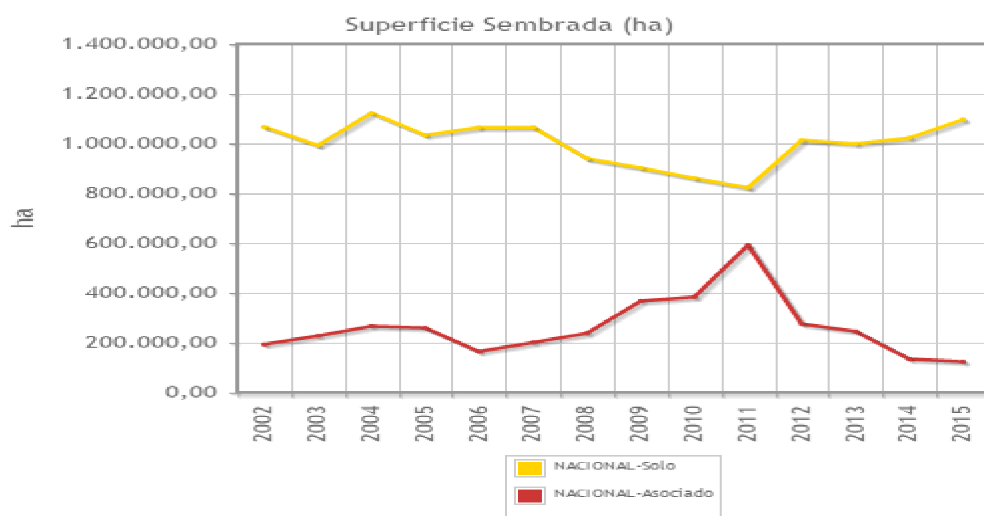

Figure 17. Transitory crops in Ecuador both alone (yellow line) and associated (red line). It refers to the surface that has been planted using seeds and/or seedlings. Source: ECUADORENCIFRAS.

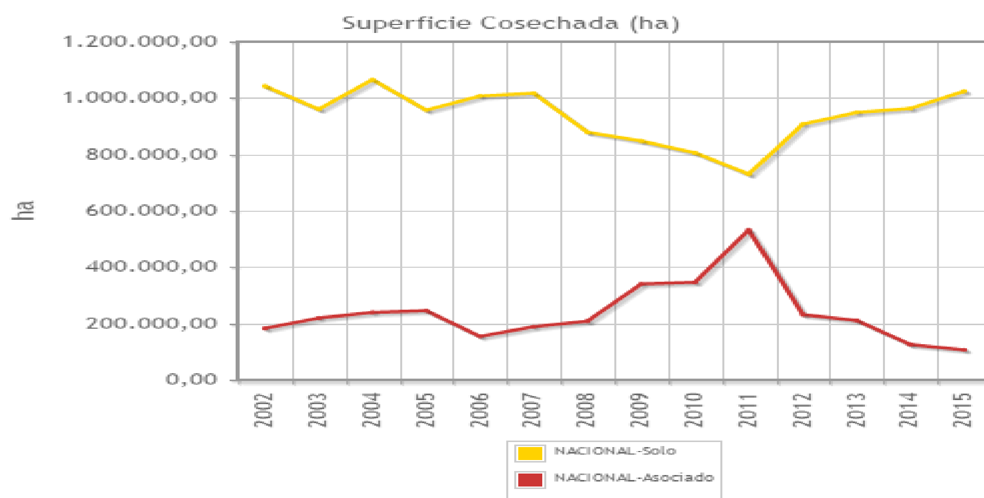

Figure 18. Transitory crops, harvested area. Source: ECUADORENCIFRAS.

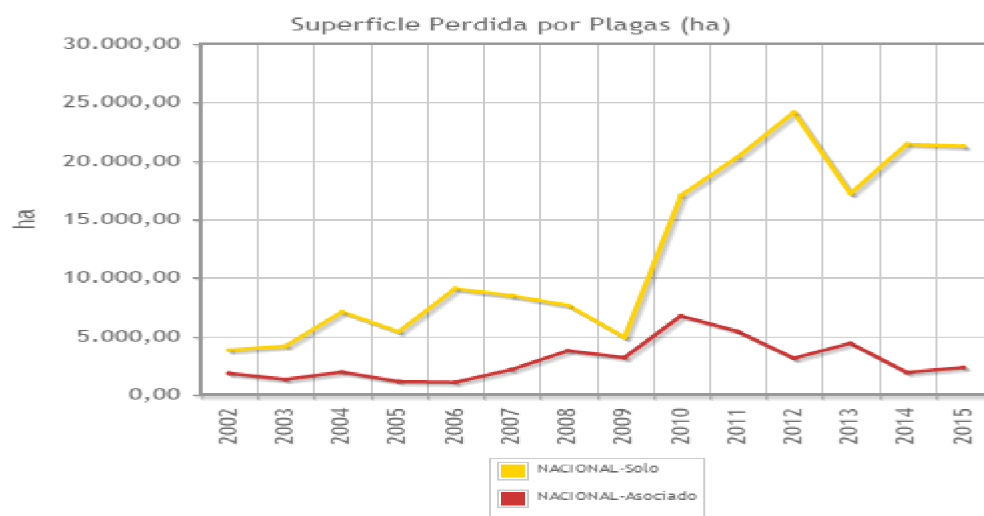

Figure 19. Transitory crops, lost surface by pests. Source: ECUADORENCIFRAS.

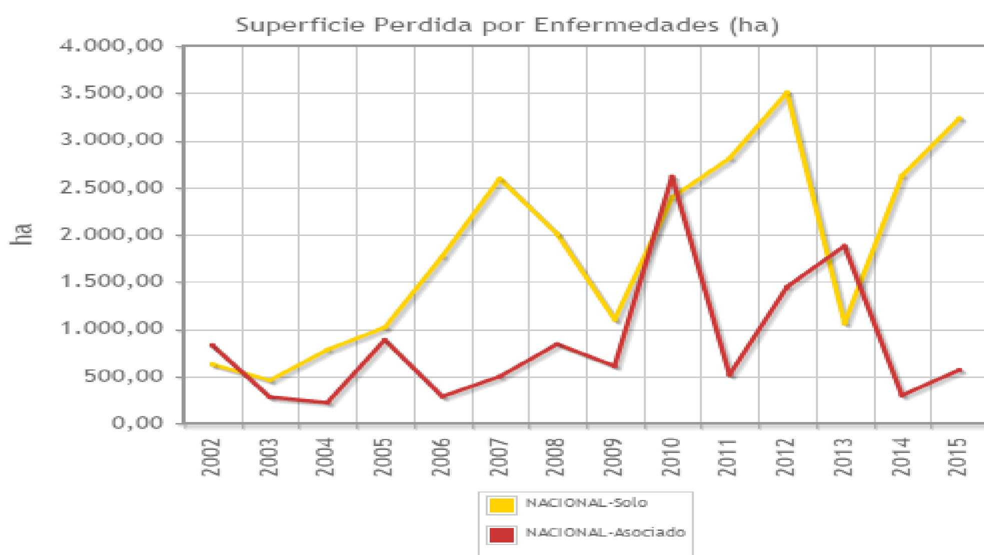

Figure 20. Transitory crops, lost surface due to diseases. Source ECUADORENCIFRAS.

2.3 PLAGUES THAT AFFECT TOMATO CULTIVARS

There are many pests that can attack tomato cultivars, bacteria, fungi, nematodes, virus, insects, mites, and vertebrates. The appearance of these can be influenced by environmental conditions, season and soil characteristics. (California, Integrated Pest Management for Tomatoes, 1998)

2.3.1 Cutworm (*Agrotis sp.*)

To feed the larvae it eats the plants neck causing a rupture, especially during transplantation time. The soil is a refuge for adults, dry season and presence of weeds favor their reproduction.

2.3.2 Mite (*Symmetrischema plaesiosema*)

The larvae feed on the bark of the plant, where they let galleries that prevent sap passage followed by plant death. The soil is a refuge for adults, especially when tomato is cultivated or when storing tomatoes. The adult is a brown butterfly with wings folded to the body. Living more or less 20 days, copulates at night, flies short, low and blends. Around 180 to 235 eggs are positioned between plant necks.

The larvae are transparent, whitish, dark brown head, and then they turn green and purple in the bottom, measuring from 1 to 1.5 cm, with black spots on the body. Remaining 15 to 18 days in the soil as pupae. Moths can have 8 to 10 generations in a year.

Favorable conditions for its development are dry, hot weather, and the presence of weeds.

2.3.3 Fleam (*Aphis sp.*)

Small insects ranging in colors from black, green, or gray, according to the species; located in tender buds, sucking the sap and transmitting virus.

They multiply on a large number of herbaceous and shrubs plants that serve as host, then access to tomato.

The biological cycle lasts 7 days, the fecundity of females is estimated at 30 to 40 individuals per generation.

Dry and warm weather, and the presence of weeds encourage their reproduction.

2.3.4 Acarus (*Aculops lycopersici*)

Stick their stilettos and absorb cellular juices emptying the cells. Affected tissue acquires a brown coloration also stems and fruits. In severe attacks the leaves desiccate reducing plant development.

Host plants are a large number of herbaceous plants, shrubs and trees where multiplication occurs, moving then to tomato plants.

Its biological cycle lasts 6 to 7 days at temperatures near 27 ^{o}C and 30% relative humidity.

Above 40 ^{o}C high mortality occurs, which limits their multiplication. Below 12 ^{o}C development is interrupted.

2.3.5 White fly (*Trialeudores vaporariorum*)

Flies absorb cell juices, with high populations causing yellowing and drying of leaves. Oily black spots appear on the lower leaves and fruits, which are product of the fly dregs, unfolding the appearance of sooty mold which prevents leaf transpiration and causes death.

They multiply on a large number of herbaceous plants and shrubs and trees, which serve as host to access tomato plants.

The eggs pass to nymph state from 7 to 15 days, nymph mature in about 20 days, taking additional 8 days to become adult.

Favorable development conditions are temperatures between 20 to 25 ^{o}C and 80% relative humidity. Below 10 ^{o}C development is interrupted.

2.3.6 Leaf miner (*Liriomiza spp.*)

Galleries appear inside the leaves, when opened a greenish larva 4 to 6 mm in length can be found. It also attacks and destroys flower clusters, fruits, axillary and terminal buds. Severe attacks can cause partial or total crop destruction.

Temperatures between 20 to 25 ^{o}C and relative humidity of 40% favor its development.

2.3.7 Late blight (*Phytophthora infestans*)

Oily spots appear on the leaves, drying in the center and fading in the outline. Some ribbing turn brown. Mottled brown spots appear on infected fruits, occasionally a white powder cover small fruits.

The fungus multiplies rapidly and is able to infect many plants.

It remains in plant debris on the ground and spreads through the rain and wind, the fungus penetrates through the stomata.

Favorable conditions for its development are high relative humidity above 90% accompanied by temperatures of 10 to 25 ^{o}C, moderately cold nights and warm days.

2.3.8 Early blight (*Alternaria solani*)

Symptoms are concentric small and brown spots on the leaves, small elongated brown spots on the stem, the central part is gray. Dark brown spots in fruits, necrotic sepals and consequent flower fall.

It remains in the soil and on plant debris, propagates through the wind and rain.

High relative humidity 80% and temperatures between 18 and 20 ^{o}C favor its development. Condensation of water in surrounding environments cause the disease to progress rapidly. Plants with many fruits are the most sensitive.

2.3.9 Powdery Mildew (*Oidium sp.*)

The leaves and stems are covered with a whitish powder. Affected leaves turn yellow and then dried. In severe attacks flower clusters, leaves and even plants are lost.

The fungus multiplies quickly and is able to attack and colonize numerous plants.

It is preserved in cultivated plants and weeds.

It spreads through the rain, wind and people working in the area.

Favorable conditions for its development are lower relative humidity 60% accompanied by temperatures of 17 to 30 ^{o}C. Dry periods allow maturation and spores dispersion.

2.3.10 Gray mold (*Botrytis sp.)*

Presence of concentric rings on leaves. Gray mold on leaves, flower clusters, fruits and stems. White or yellowish rings on fruits. The newly transplanted plants have a light brown constriction in the plant neck, presence of dry tissue.

It multiplies rapidly, attack and colonize many plants, especially in aged tissues wounds that constitutes the nutritional basis for its development.

It is preserved in plant debris and soil in various forms, conidia, mycelium, sclerotia.

It spreads through the rain, wind and people working in the area.

Favorable conditions are high relative humidity of 95% accompanied by temperatures of 17 to 23 ^{o}C.

2.3.11 E. Fusarium (*Fusarium oxysporum*)

This disease affects the roots and neck in the plant, and can go up to 30 centimeters above the neck. General wilting of the plant, dark brown interior stem, browning of neck central cylinder and root, base of the plant roots are rotted.

Spores are conserved in numerous substrates, soil, and plant debris.

It spreads through water irrigation, rain, drafts and contaminated plants.

Favorable conditions for development are temperatures between 18 and 26 ^{o}C. Water stress, accompanied by an excess of water favors the attack on the roots. This fungus is able to quickly colonize soils or recently disinfected substrates.

2.3.12 F. Erwinia (*Erwinia carotovora*)

Symptoms are stem browning, yellowing foliage, and wilting plants.

It remains in the soil and on diseased plants.

It is spread through irrigation water.

These are bacteria that develop under high hydrometric conditions and temperatures ranging from 5 to 37 ^{o}C. High humidity increases the severity of the attack on stalks. Bacteria are mobilized to wet plant surfaces, over 90% relative humidity the spread accelerates.

2.3.13 Anthracnose (*Colletotrichum coccodes*)

Early symptoms appear on ripe fruit as depressed circular lesions 1.2 cm in diameter, as they age became dark and dotted with small black specks. In field crops it attacks the root cortex becoming dull, brown and gradually decomposing, otherwise turning discolored and decomposing. It is most common on greenhouse tomatoes, present in many soils with poor crop rotation and where sensible crops are grown frequently.

It develops over a broad range of temperatures, with an optimal of 22 ^{o}C, although germination is strongly slowed down at temperatures below 10 ^{o}C. Climatic condition influences the germination of conidia which occurs at temperatures between 15 to 30 ^{o}C. Temperatures greater than 38 ^{o}C inhibit the fungal development.

High humidity on plants and soil, caused by rainy periods or irrigation encourage anthracnose, also salinity predispose plants to the fungus attack. (Cañar, 2003)

2.4 INTEGRATED PLAGUE MANAGEMENT IN TOMATOES

Integrated pest management IPM is an approach that allows pests control by considering pathogens and their relationship with the environment as a part of the crop production, without relaying only in pesticides and bringing together different tactics in one program, in a safe and effective way.

One of the objectives of IPM is keeping pests levels below an economic threshold, keeping a balanced ecosystem where the species can coexist without pest domination. In fact, agrochemicals could kill some beneficial insects that consume pests along with the pathogens therefore depleting natural defence mechanisms.

In order to have a good IPM program, pest populations should be identified and measured, knowing the levels of damage and possible strategies that could be implemented to improve the effectiveness of pest control. It is interesting to note that some agrochemicals are no longer effective considering pest resistance development.

One of the goals would be the replacement of agrochemical pesticides by less harmful substances, with an environmental friendly approach. Many agrochemicals can persist in the environment and also in organisms, leading to significant contamination of water sources. (Secretaria de Agricultura, 1998) When pesticide use cannot be avoided, the best choice should be products that cause the minimum damage on other organisms.

With the aim of estimating control and prevention methods, some points should be taken into consideration.

Field selection helps to visualize if the soil will be suitable for planting, considering the water holding capacity and soil composition. The mechanism of good agricultural practices allows knowledge of the land history and activities developed in neighboring lands.

Soil preparation allows proper consistency to permit good water drainage and suitable clod size for root developing.

Soil solarization is a method that uses a plastic coating applied directly on the ground, increasing soil temperature to kill some pests like soilborne pathogens and weeds. This approach is however limited when production extensions are big. Drip irrigation lines would be the most desirable and should be placed under the plastic. To increase the useful life of the plastic film, UV resistant material is available.

Seedbed preparation can be performed taking into account that seeds should not produce plants with high density, since competition would eliminate some of the plants. When transplanting, the soil must be ready and free of weeds; special care should be taken in handling plants to prevent viral diseases spread.

Different irrigation systems can be used according to soil texture, each one has specific considerations. In furrow irrigation it is necessary to pay attention not to flood the soil as it encourages the development of pathogens such as fungi. Sprinkler irrigation is useful when seeds have been planted, but can promote the dispersion of bacteria. Drip irrigation also allows the dosage of fertilizers. In any of the strategies used, constant monitoring of soil moisture is required. (California, Integrated Pest Management for Tomatoes, 1998)

The main purpose of integrated pest management system is the prevention, suppression and eradication of pests. Prevention permits getting ahead of possible scenarios that could arise pathologies, taking into consideration all factors involved in planting. The pests suppression does not always look for their total elimination but to lower populations to tolerable levels not impacting heavily on the economy. The eradication process seeks to eliminate pests presence in a crop field, this system is used especially when pests have already been introduced. (Secretaria de Agricultura, 1998)

2.5 CONVENTIONAL PEST CONTROL IN TOMATOES

Analyzing control programs established by companies that provide services to the ecuadorian agricultural sector, one of the most used are pesticides. Depending on the production areas, programs vary as a result of different climate and altitude characteristics.

According to the Food and Agriculture Organization of the United Nations FAO, pesticides are defined as any substance or mixture of substances intended for

preventing, destroying or controlling any pest, including vectors of human or animal diseases; unwanted species of plants and animals that are causing harm or interfere in any way in production, processing, storage, transportation or marketing food, agricultural products, wood and wood products or food animals, or which may be administered to animals to combat insects, arachnids or other pests in or on their bodies.

Inorganic products in agrochemicals are composed of different elements, such as arsenic, mercury, copper, boron, sulfur, etc. Most have fallen into disuse or have been banned, but some are still in use such as copper oxychloride and sulfur.

Most of today used pesticides belong to the group of synthetic origin, such as organophosphates, carbamates, pyrethroids, organochlorines. Organophosphates and carbamates cause nervous system imbalances in insects by blocking the acetylcholinesterase (AChE) enzyme which degrades the neurotransmitter acetylcholine. Prolonged acetylcholine residence in cholinergic synapses results in hyper-excitation and eventual death. The resistance mechanism is presented by insensitivity to cholinesterase. (Ishaaya, 2000)

Other mechanisms by which insects become resistant to agrochemicals are target-site when the insect could be genetically changed; barrier modifications in the outer insect cuticle reducing toxins absorption; behavioral resistance when the insect may recognize the danger and avoid it. (IRAC, 2016)

Organophosphates and pyrethroids block the transmission of nerve impulses, thus membrane potentials are altered by interfering with sodium channels; this functional imbalances produce insects death. Insensitivity is caused by failure of the coupling mechanism avoiding the insecticide to block the system. (Jaramillo, 2007)

Pesticides can be classified according to the pest type they control:

• Acaricides: to control mites.

• Attractants: used to attract pests (usually traps).

• Defoliants: cause leaves falling without killing the plants.

• Fungicides to control diseases caused by fungi.

• Herbicides to control weeds.

• Insecticides used to control insects.

• Nematicides: to control nematodes.

• Physiological regulators: accelerate or retard growth, stimulate flowering or fruiting or change in any way the behavior normal plants.

• Repellents: used to ward off pests.

• Rodenticide: to control rodents like rats.

• Slimicides: to control slugs and snails.

• Soil disinfectants: products that control almost all organisms living in the soil, such as fungi, weeds, insects and nematodes.

According to the way of action, insecticides can be classified in: contact, ingestion and inhalation.

According to the movement in the plant: surface, translaminar and systemic.

In relation to selectivity when interacting with beneficial fauna, they can be classified in selective and non-selective.

By their mobility in the plant fungicides are classified in contacts or systemics. Contact fungicides are also called protectants as they remain in the surface of the plant. Systemic fungicides are called penetrants and they are absorbed by the plant. By their role of protection they can be preventive or curative. By the breadth of activity fungicides can be single-site or multi-site, according to their action on one point or several points of metabolic pathway. By their mode of action, they can elicit the systemic acquired resistance (SAR response) from the host plant. By their breadth of activity: narrow-spectrum or broad-spectrum considering the range of pathogens closely related and unrelated. According to the type of chemical of its formula they can be inorganic or organic. (APS, 2016)

Reviewing the available information for a tomato phytosanitary program from Bayer, the following products are recommended: Triflumuron (larvicide), Flubendiamide (benzenedicarboxamide, phthalic acid diamide), Pyrethroid (Beta-cyfluthrin), Imidacloprid, Chloronicotinyl, Microbial, B.t.k. strain ABTS-351, Espirotetramate, Deltametrine. For fungus caused diseases: Propineb, Fenamidone, Trifloxystrobin, Tebuconazole, Tebuconazole/Fluopyram, Thiuram,

Propamocarb/Fosetyl, Anilinopyrimidine. To control weeds: Fluazifop-P-butil, Oxadiargyl, Metribuzine. (Bayer, 2014)

Paute is a valley located northwest of Cuenca city in Ecuador, with an altitude of 1,850 m.a.s.l., with average temperatures of 18 ^{o}C and 60% relative humidity. Studies on the use of pesticides in greenhouse tomato crops indicate that chemicals mainly used are: Captan: (N-trichloromethylthio-4-cyclohexene-1,2-dicarboximide); Bala: S,S'-(2-dimethylamino-trimethylene) bis(thiocarbamate); Curzate m8: 1-[(EZ)2-ciano-metoximinoacetil-3-etilures]; Cabrio top: Methyl N-(2-{[1-(4-chlorophenyl)-1H-pyrazol-3-yl]oxymethyl}phenyl)-N-methoxy carbamate; Curacron 500: O(4- bromo-2-clorophenyl)-O-ethyl-S-propyl phosphorothioate; Ridomil: (R,S)-2-[(2,6-dimethylphenyl)-methoxyacetylamino]-propionic acid methyl ester. There is a tendency for farmers to apply agrochemicals to tomato crops as often as they consider necessary, without reflecting on damages to the crop, environment and final consumer. (Reinoso, 2015)

The residual presence of some organo phosphorus insecticides in different matrices such as water, oil, milk, palm oil, summer flowers has been demonstrated. Insecticides found are: lindane, BHC, heptachlor, DDT o-p and p-p DDT, aldrin, chlordane, heptachlor epoxide. (Ambiente, 2004)

Chemicals mentioned above are contained in the list of banned pesticides in Ecuador by the Ecuadorian Agency for Agro Quality Assurance in 1992. Currently there are about 2,000 registered pesticides and related products that correspond to broad-spectrum and persistence insecticides, consisting in extreme and highly dangerous categories. The list of forbidden pesticides includes: Aldrin, Dieldrin, Endrin, BHC, Campheclor, Clordimeform, Chlordano, DDT, Lindano, Leptophos, Heptachlor, Methyl Parathion, Diethyl Parathion, Ethyl Parathion, Mirex, Aldicarb (restricted sale and use), Monocrotophos, Parathion, Metamidofos, Fosfamidon, dry powder formulations with 10% mixture or more of Carbofurane, belonging to the most dangerous toxicological category, Ia and Ib. Ecuador forbids aerial application, however, it is permited to sell them under the prescription of an agronomist. (Agrocalidad, 2016)

Tomato is one of the most exposed crop to chemicals, as long as it is vulnerable to many pests and to ensure good production yield is constantly fumigated. Some agrochemicals are considered carcinogenic and although they have been banned

in the past decade, still appear in some foods, as it was demonstrated by the study of AGROCALIDAD (Agrocalidad, 2016), the National Authority in Ecuador for plant and animal health responsible for the definition and implementation of control policies and regulations for the protection and improvement of animal health, plant health and food safety.

In recent years there is concern for the increase in poisoning statistics related to the use of agrochemicals (insecticides, fungicides, herbicides, nematicides) or their mishandling. Studies in the northern of Ecuador, in Carchi province, where large fields of potatoes are grown show that indiscriminate use of agrochemicals is the second leading cause of death by poisoning in the province. The use of pesticides in Ecuador began in the 50s, followed by land reform in the early 60s and late 70s, parallel to a loss of ancestral knowledge about fertilization, soil management, seed cultivation and production. Currently, most farmers do not believe that it is possible to produce without fertilizers or pesticides. (Bellettini & Ordoñez, 2013)

The Information and Toxicological Advice Center CIATOX that belongs to the Ministry of Public Health in Ecuador, presents comparative data from years 2009, 2010 and 2011, showing that poisoning have risen by 40%. Data from 2011 state that the use of pesticides contributes to 49.2% of the total, and within this, organophosphate insecticides constitute 17%. The poisonings can be intentional 62% and accidental 37%. In reference to occupational poisoning type, 30% are caused by products misuse, 37% for not using personal protection, 33% were incidents and accidents. (Publica, 2008)

CHAPTER 3

STRATEGIES TO CONTROL PESTS WITH PLANTS

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3. STRATEGIES TO CONTROL PESTS WITH PLANTS

3.1 BIOPESTICIDES

A pesticide is defined as a substance or a group of structurally similar substances intended to prevent, destroy, repel, or mitigate any pest, or function as a plant regulator, desiccant or defoliant, and any nitrogen stabilizer. (Rechcigl & Rechcigl, 1999)

The use of alternatives to chemical pesticides, which are effective for pest control in agriculture, can be achieved through biopesticides, taking into account several factors as the increased environmental awareness and benefits in the consumption of organic products, lower toxicity, safety and high efficiency for pest control. (PRESSWIRE, 2012) It is expected that biopesticides market will have a strong growth until 2020, projected to generate USD 6.6 billion, with a compound annual growth rate (CAGR growth) of 18.8%. In fact, to capitalize this growth trend several chemical companies that lead crop protection are rushing to develop and manufacture biopesticides. (WIRE, 2016) Although biopesticides worldwide sales are expected to exceed USD 1 billion per year it is still small compared to USD 35 billion in the worldwide pesticide market. (Cline, 2009)

As Waage notes (Waage, 1997): “Biopesticides, being living organisms, have properties which make their design, production and use potentially very different from that of chemical pesticides”. The existing regulation system comprise industry manufactured chemicals, thus new legislation for organic products should be considered.

Biopesticides are considered a green product, being derived from living organisms have some advantages when compared with chemical products: are less harmful than those of chemical origin, more target specific, usually are more effective in small amounts, break down more rapidly without leaving problematic residues. The use of biopesticides is an important component of integrated pest management system IPM. (Shukla & Shukla, 2012)

Biopesticides can be classified into three categories according to their origin: (Agency U. S., 2016) (Leahy, Mendelsohn, Kough, Jones, & Berckes, 2014)

1. Biochemical pesticides, naturally occurring substances that control pests by nontoxic mechanisms. Interfere with mating, attract insect pests to traps, also called semiochemicals. Includes plant products such as essential oils, compounds synthesized by other organisms like chitosan and chitin.
2. Microbial pesticides: microorganisms like bacteria, fungi, oomycetes, virus and protozoa. Each separate ingredient is relatively specific for its target pest or pests.
3. Plant incorporated protectants or PIPs: use of recombinant DNA technology allowing the plant to produce pesticidal substances.

In the US Environmental Protection Agency (EPA), biopesticides are grouped with other low-risk substances and listed along with mineral substances like kaolin and iron phosphate, which do not make much sense when analyzing their origins, living origin for biopesticides and inorganic for minerals. (Bailey, 2010)

Biopesticides can have multiple modes of action: antibiosis, parasitism and predation, competition, contact inhibition, induced resistance either systemic acquired resistance (SAR) or induced systemic resistance (ISR). (Tocci, 2014)

The use of bioproducts is an alternative to agrochemicals, farmers would not need to use environmental harmful and costly chemicals. The ancient wisdom of traditional cultures around the world allows to foreknowledge a large number of plants that have been used in mixtures or alone for pest control.

Most plants contain substances that can be exploited for biological control as a result of its compounds toxicity. The fact of its natural origin should not disregard precautions during preparation and application. Some recommendations that must be considered are: avoid skin contact and inhalation by using personal protection, allocate containers that are unique to organic pesticides, do not consume products that have been recently fumigated with bioinputs.

Insecticidal compounds can be isolated from plants by extraction and distillation procedures. Among the regions with history of plants traditional use as pesticides are: Asia, Central America, India, South America, and Iran. (Rechcigl & Rechcigl, 1999)

The following table, Table 3, features the main characteristics of major botanical insecticides.

Table 3. Main characteristics of major botanical insecticides. Source Hayes, Worthing and Walker, Ware.

Botanical insecticide	Source plant(s)	Mode of action & toxicity*	Uses
Pyrethrum/ Pyrethrins	Flowers of the pyrethrum daisy, *Chrysanthemum cinerariaefolium*	Interferes with Na and K ion movement in nerve axons. Mammalian oral and dermal LD_{50}s greater than 1000; some allergic reactions can occur in humans and other mammals.	Many. On pets and humans to control fleas, ticks, lice. Used with synergists as aerosol "bombs" in homes and food plants. Breaks down very rapidly. Mixed with more stable botanicals for field and garden uses.
Rotenone	Roots of *Derris*, *Lonchocarpus*, other tropical legumes	Disrupts energy metabolism in mitochondria. Mammalian oral LD_{50}s range from 25–3000, dermal >1000. More acutely toxic to mammals and more persistent than many botanicals. Some chronic toxicity suspected. Extremely toxic to fish.	In gardens and orchards against many insects, especially beetles. Persists at effective levels for 3 to 5 days or more. Used purposefully as a fish poison.
Sabadilla	Seeds of the tropical lily *Schoenocaulon officinale* and European *Veratrum album*	Interferes with Na and K ion movement in nerve axons. Mammalian oral LD_{50}s near 4000. Irritates skin and mucous membranes; potent inducer of sneezing.	In vegetables and fruits, particularly against squash bug, harlequin bug, and citrus thrips. Breaks down very rapidly.
Ryania	Woody stems of *Ryania speciosa* (S. American woody shrub)	Activates calcium ion release channels and causes paralysis in muscles of insects and vertebrates. Mammalian oral LD_{50} near 1000; dermal near 4000. More persistent than rotenone but less potent.	In fruit and field crops, particularly against caterpillars and thrips. Often combined with rotenone and pyrethrins in commercial mixtures for garden use.
Nicotine	Tobacco, other *Nicotiana* species, also *Duboisia*, *Anabasis*, *Asclepias*, *Equisetum*, and *Lycopodium*	Mimics the neurotransmitter acetylcholine and overstimulates receptor cells to cause convulsions and paralysis. Mammalian oral LD_{50}s range from 3–188; dermal 50 or lower. **Nicotine insecticides are very toxic to humans.**	Mostly in greenhouses and organic gardens. Free nicotine fumigations target aphids, thrips, and mites. Nicotine sulfate in nonalkaline solutions may last 24 to 48 hours and give limited residual protection.
Neem/ azadirachtins	Leaves, bark, and seeds of neem (*Azadirachta indica*) and Chinaberry (*Melia azedarach*)	Biochemical nature of feeding deterrence, repellence, and growth regulation effects are not well described. Mammalian oral LD_{50} greater than 13,000; used medicinally in humans.	On many crops and landscape plants; especially against soft-bodied and sedentary pests. Very short persistence on treated plants.
Limonene/ Linalool	Citrus oils (linalool is also present in many other plants)	Limonene: mammalian oral LD_{50} > 5000; linalool: mammalian oral LD_{50} > 2400; dermal > 3500. Limonene causes spontaneous stimulation of sensory nerves; biochemical nature of modes of action are not well described for either. GRAS, but chronic toxicity suspected for limonene.	Mostly in pet shampoos, dips, and sprays to kill fleas and ticks. Synergized by PBO. Very short persistence on treated surfaces.

* LD_{50} estimates are expressed in mg of toxin per kg of body weight for test animals. LD_{50} = dose estimated to kill 50% of the test animal population; higher numbers indicate lower toxicity. Sources: Hayes (1982); Worthing and Walker (1987); Ware (1988). See text for more details for all columns.

3.2 PLANTS COMMONLY USED TO PREVENT AND TREAT PESTS

The ancestral use of plants in Ecuador is linked to its population and cultural traditions, being Ethnobotany a science which studies how each population uses the existing flora in their environment. Unfortunately deforestation practices have placed in disappearing danger many native plants and therefore the possibility to use them in different medical and ecological farming practices will be lost. "In Ecuador, ethnobotany has awakened a consciousness of change in the new generations of scientists and academics. The challenge facing Ecuadorean ethnobotanist in the 21st Century is to demonstrate the feasibility of a sustainable use of its plant diversity through traditional practices involving the participation of local peoples who have a profound respect for nature since they conceive it in a holistic manner". (Rios, 2007)

The book "Useful Plants of Ecuador" by Montserrat Rios et al., describes 3,072 existing plants in various ecological regions of Ecuador, which are used in different ways like medicine, food, building materials, among others. Ethnobotanical knowledge has accumulated over generations, and it tries to explain the relationship between humans and plants.

Local governments in some countries have published agriculture manuals listing appropriate plants and suggesting preparations use. Biological products that help the production of vegetables are knowns as bio-stimulants, bio-fertilizers, bio-fungicides, bio-insecticides and bio-repellents. (FAO, Los Biopreparados para la Produccion de Hortalizas en la Agricultura Urbana y Periurbana, 2013) Products from plants, fish and animal liquid extracts, which serve as insecticides and fungicides can be used to treat diseases in farm animals and as energy supplements for humans. (CENTER, 2006) (ProDeSoC-IPADE) (ECOTENDA) (Quiros, 2004) (Funsalprodese, 2000) (Agricultura, 1997) (Hernandez, 2000)

The following is a list of some useful plants and products:

- Pumpkin (Zapallo, *Cucurbita maxima*): seeds are soaked in 70% alcohol and used as an insecticide against cockroaches.
- Wormwood (Ajenjo, *Artemisia absinthium*): infusion of its leaves control slugs in crops and fleas on animals.

- Basil (Albahaca, *Ocimum basilicum*): repellent insecticide, miticide, controls moths, aphids, spiders.
- Mugwort (Artemisa, *Artemisia vulgaris*): toxic for animals, prevents or restricts the passage of soil insects.
- Barbasco (Barbasco, *Lonchocarpus nicou*): powerful insecticide, controls external parasites.
- Calendula (Calendula, *Calendula officinalis*): control nematodes and whiteflies.
- Onion (Cebolla, *Allium cepa*): controls pest larvae in different crops.
- Dandelion (Diente de Leon, *Taraxacum officinale*): nematicide when planted in soils with nematodes.
- Canavalia bean (Frejol de Canavalia, *Canavalia ensiformes*): controls carrier ant and acts as a fungicide.
- Fresh chamomile (Manzanilla, *Matricaria chamomilla*): fungicide to control fungi that cause root and neck rots, and mildews in different plants.
- Stinging nettle (Ortiga, *Urtica dioica*): accelerates the decomposition of organic material for composting. Its infusion stimulates plant growth and controls caterpillars, aphids and nematodes.
- Horseradish (Rabano picante, *Armoracia rusticana*): controls worms in all crops.
- Rue (Ruda, *Ruta graveolens*): strong odor attracts flies and black moths without causing problems in crops.
- White sage (Salvia, *Salvia apiana*): it repels whitefly and fleas.
- Tobacco (Tabaco, *Nicotiana tabacum*): controls thrips, whiteflies, fleas, and mites.
- Melissa (Toronjil, *Melissa officinalis*): Repels fleas, moths and aphids.
- Spearmint (Yerbabuena, *Mentha spicata*): controls sucking insects such as lice and aphids.
- Chilli Pepper (Aji, *Capsicum annuum*): exerts insecticide and repellent action.
- Garlic (Ajo, *Allium sativum*): control and repel aphids, bugs, flies, mosquitoes, nematodes, fungi and bacteria.
- Horsetail (Cola de Caballo, *Equisetum arvense*): used as a fungicide to controll fungi in Solanaceae.

- Sunflower (Girasol, *Helianthus annuus*): insecticide to repel flies.
- Fern (Helecho, *Pteridium aquilinum*): control mites, aphids and mealybugs.
- Castor Bean (Higuerilla, *Ricinus communis*): flies and mosquitoes repellent.
- Mamey Zapote (Mamey, *Pouteria sapota*): insect repellent.
- Mango (Mango, *Mangifera indica*): mosquito repellent.
- Neem (Neem, *Azadirachta indica*): a broad spectrum insecticide, fungicide and nematicide.
- Papaya (Papaya, *Carica papaya*): control fungi, rusts and mildew.
- Propolis (Propoleo, bee product): liquid organic fungicide in the form of water-alcohol solution based on natural propolis.
- Red thyme (Timo, *Thymus vulgaris*): antifungal and antimicrobial activity.
- Citrus seeds (Semillas de Citricos): proven effectiveness, algaecide, fungicide and bactericide.
- Natural pyrethrin (Piretrina, *Pyretrum cinerariefolium*): insecticide obtained from pyrethrum dried flowers, characterized by its rapid contact action, causing paralysis in aphids, whiteflies, mites, etc.
- Orange peels (Cascaras de naranja): insect control.
- Ginger (Jengibre, *Zingiber officinale*): repellent, insecticidal.

3.3 NATURAL EXTRACTS PREPARATIONS

Extracting operations are used to separate active substances from different types of matrix using suitable solvents that match the chemical characteristics of substances to be separated, in standard extraction procedures. The products thus obtained are not pure products and should undergo further processing if the objective is to obtain single compounds. (ICS-UNIDO, 2008)

Some of the methods listed below require appropriate equipment and qualified personnel, while others are general methods that can be easily done and also have been used for years in many countries around the world.

Many types of preparations can be made depending on the methodology used: infusions, decoctions, fluid extracts, tinctures, semisolid and powdered extracts. The products obtained from plants are relatively impure liquids, semisolid or powders that can be used in different formulations.

3.3.1 SIMPLE EXTRACTION OR MACERATION

The plant product, whole or coarsely powdered, is placed in a suitable container in contact with the solvent, at room temperature with or without stirring, protected from light, for a period of at least three days. Filtration is required in order to separate the liquid which has the compounds that have been extracted.

3.3.2 DIGESTION

It is a maceration form in which low to moderately high temperature is used to facilitate removal in order to increase the extraction efficiency.

3.3.3 CONTINUE EXTRACTION OR PERCOLATION

This procedure is widely used in galenic pharmacy to obtain ingredients in the form of tinctures and fluid extracts. Plant material is mixed with a suitable solvent and introduced into a container that has both ends opened, like conical extraction funnels.

An initial maceration is allowed for varying times from 4 to 24 hours, then the lower key percolator is opened allowing liquid to drip slowly. Small amounts of solvent should be poured through the upper opening to replace the liquid that has percolated. The obtained liquid can be finally decanted or filtered.

3.3.4 INFUSIONS

This method allows the extraction of vegetable materials by a liquid which can be cold or heated near the boiling point, then the plant material is placed and left to stand for a variable time. Finally it is filtered to obtain a clear liquid.

3.3.5 DECOCTION

In this process the plant material is boiled together with a certain amount of water, especially for the extraction of thermostable compounds. Subsequently, the liquid fraction can be concentrated evaporating to a smaller volume.

3.3.6 CONTINOUS EXTRACTION OR SOXHLET EXTRACTION

This equipment allows continuous extraction by refluxing, the solvent placed in the lower container evaporates by heat and the condensate falls into the upper container in which the plant material is placed inside a cellulose or filter cartridge. Once a volume covering the cartridge is reached, the liquid content is syphoned off and returns to the lower container. This procedure is repeated during a variable time in hours. The advantage lies in the removal of large quantities of active ingredient with small amounts of solvent. The use of special solvents is a limiting factor for the process in some cases.

3.3.7 AQUEOUS ALCOHOLIC EXTRACTION BY FERMENTATION

The plant material is mixed with liquid (previously boiled) for a long period of time during which spontaneous fermentation occurs producing alcohol which serve both as a preservative and extraction media. In traditional medicine different container materials has been used such as wood, porcelain, metal, and clay.

3.3.8 SUPERCRITICAL FLUID EXTRACTION SFE

SFE is a process to separate a component from a matrix, using supercritical fluids as the extracting solvent, for example carbon dioxide CO_2, and sometimes ethanol or methanol. This is an intermediate state between gas and liquid that greatly increase the solvent capacity aided by pressure and temperature above the critical point. The viscosity is much lower than that of liquids. Very low surface tension allows high penetrability through solids and greater diffusivity in liquids

3.3.9 COUNTER-CURRENT EXTRACTION

Separate components of a plant matrix are pulverized to produce a fine slurry, which is extracted by moving phases in opposite directions but in continuous contact, usually a cylindrical extractor that connects the plant sample with the solvent. The extraction can be optimized using less solvent, and it is more effective compared to other traditional methods.

3.3.10 ULTRASOUND EXTRACTION (SONICATION)

Ultrasound with frequencies ranging from 20 to 2000 kHz generates an effect of gaseous cavitation explained by the generation of microbubbles in a liquid medium, which when reaching a critical size can implode raising the instantaneous temperature and affecting the microenvironment cellular structure increasing cell walls permeability, even if the temperature of the treated liquid does not rise substantially.

3.3.11 MICROWAVE EXTRACTION

It is an alternative method for essential oil extraction, using conventional methods as hydro distillation or extraction without solvent. To carry out this technique a conventional microwave oven can be modified.

3.4 NATURAL EXTRACTS PREPARATION IN THE PRESENT WORK

In order to determine the genetic response of tomato plants, various aqueous plant extracts were tested, which were then narrowed according to laboratory obtained results. Analyses of existing literature about plants uses were taken into account, also experiences of people who cultivate the land ancestrally. Plants for aqueous extracts were bought in local markets of Cuenca city in the south of Ecuador, where they are regularly used for food preparation or for traditional medicine.

3.4.1 RUE, *Ruta graveolens*

Ruta graveolens L. belongs to the family Rutaceae, is a characteristic evergreen shrub present throughout the ecuadorian territory. Traditional medicine in Ecuador uses it as a medicinal plant to relieve colic, treat pneumonia and rheumatism, but is also used by ancient sorcerers as a ritual plant because rue smell drives away evil spirits and clean spells in a person. (Rios, 2007)

Rue has a generous amount of essential oil with very pungent odor because of terpenes composition as limonene and pinene found in the leaves and flowers,

also contains flavonoids, among the most striking is the rutosid, it has some alkaloids that could be antimicrobial agents, coumarins, tannins and bergapten.

3.4.1.1 STUDIES ON NATURAL EXTRACTS OF RUTA GRAVEOLENS

The bactericidal activity of crude extracts from 84 species of plants were evaluated, including *R. graveolens* both *in vitro* and *in vivo* against bacterial leaf spot seed-borne xanthomonads. Tomato seeds infected with *Xanthomonas perforans* were treated with crude extracts. Treatment of tomato seeds had a positive effect in terms of antibacterial activity without inhibiting effects on plant development. (Mbega, 2012)

Alcoholic and etheric extracts of *R. graveolens* have been tested for bacterial inhibition against Gram + and Gram - strains showing positive results, while in ethanol mixtures results are moderate. (Ivanova, 2005)

Hydro alcoholic extracts of rue leaves obtained by hydrodistillation were characterized showing strong activity against *Staphylococcus aureus* and *Bacillus cereus*, thus this plant can be a source of antibacterial compounds with applications in the pharmaceutical industry. (Franca & Nascimento, 2015)

Aqueous and ethanol extracts were tested for inhibition of *Botrytis cinerea* and *Colletotrichum musae*, subjected to different extract dilutions. No inhibition halos were seen with aqueous extracts; alcoholic extracts demonstrated greater effectiveness but are similar to those obtained with ethanol alone because of interference with the cell membrane. (Lopez, 2006)

R. graveolens extract was evaluated against the fungi *Fusarium solani*, *Pyrenochaeta lycopersici*, and *Trichoderma viride*. It was determined that a lyophilized rue extract at concentrations of 5-40 g/L inhibited the mycelial growth. The calculated IC50 values ranged from 4.16 g/L for *P. lycopersici*, which was the most susceptible, to 15.17 g/L for *T. viride*, *Penicillium sp.* had an IC50 of 9.04 g/L. The inhibition appeared when the concentration was identical to that of the crude extract. (Oliva, Fungistatic Activity of Ruta graveolens Extract and its Allelochemicals, 1999)

Essential oil of *R. chalepensis* was obtained by hydro-distillation, a methanol extract 10% w/v tested for effectiveness against fungi *Aspergillus niger* and

Aspergillus flavus, and the yeast *Candida albicans* ATCC 2091, grown on PDA by disk diffusion tests. Inhibition diameters were between 11 to 15 mm, showing that they have significant fungicidal effect. (Aouadhi, 2013)

Effectiveness of concentrated ether extracts obtained from *R. graveolens* seeds were tested against 4th larvae state of *Culex pipiens* in which abnormalities were produced with similar effects as the ones using insect growth regulators. (Kather, 2015)

Studies to evaluate the insecticidal capacity of eight plant species, including *R. graveolens*, showed effectiveness against *Zabrotes subfasciatus* adult forms causing 100% mortality, and also inhibiting oviposition. (Ferreira da Silva, 2016)

R. graveolens essential oil and commercial phenolic analogs were used for toxicity tests by spraying against *Sitophilus zeamais* adults, *Sitophilus oryzae*, and *Lasioderma serricorne*; comparing the activity with the synthetic insecticide Dichlorvos (2,2-dichlorovinyl dimethyl phosphate). The structure-activity relationship was evaluated according to the substituent groups position in the phenol skeleton, concluding that it does influence the effectiveness of the insecticide. (Jeon, 2015)

Determination of anti-fungal activity of isolated products from ethyl extracts of *R. graveolens* was performed in microplate assays with *Botrytis cinerea*, *Fusarium oxysporum* and *P. viticola* spores, which shows that furanocoumarins 5- and 8-methoxypsoralen had moderate activity against *Fusarium oxysporum*. A novel quinolone alkaloid was highly active against *Botrytis cinerea*. *Phomopsis* species were much more sensitive to most of the compounds, with *P. viticola* being highly sensitive to all of the compounds. (Oliva, Natural Fungicides from Ruta graveolens L. Leaves, Including a New Quinolone Alkaloid, 2003)

3.4.2 CHILLI PEPPER, *Capsicum annuum L.*

Capsicum annuum L. belongs to the Solanaceae family, it is a perennial medium sized shrub present in the mountains of Ecuador and in the Amazon. In Ecuadorian traditions the fruit is used as a condiment, it is spicy and has a pleasant taste, usually accompanied with several dishes of the country, also in folk medicine the leaves and ground seeds are used to wrap newborn children, in

order to obtain a healing effect and is also used as pain distractor caused by snakebites in which the fruit is rubbed into the wounded area. (Rios, 2007)

The fruit is rich in ascorbic acid, contains diterpenic heterosids (capsianosids) and a furostanol glycoside, carotenoids are responsible of fruit color whose content increases during maturation, the spiciness is caused by amines called capsaicinoids and the main compound of this series is capsaicin, together with vainillamide. (Bruneton, 2001)

3.4.2.1 STUDIES ON NATURAL EXTRACTS OF CAPSICUM ANUUM

Organic products were evaluated for *Frankliniella occidentalis* control in rose (*Rosa sp.*) greenhouse cultivation. The product Agroverde Repel has systemic action exerting repellency and irritation of insects by virtue of its components *Capsicum sp.* and *Allium sativum*. (Bastidas, 2012)

In order to find biodegradable organic insecticides against coffee tack *Antestiopsis orbitalis ghesquierei*, alkaloids, steroids, saponins and terpenes isolated from *Capsicum frutescens* L. were evaluated. Alkaloids showed a similar effect to pesticides control with 100% mortality; steroids with 57% mortality while terpenes have minimal activity over coffee tack. (Nsambu, 2014)

The effectiveness of *C. annuum* L. extract alone or mixed with *Artemisia annua* L. extract was evaluated against immature stages of *Pectinophora gossypiella*, the parameters studied show that larval stages, life cycle and insect life, oviposition rate and fertility were affected. Combined extract of 70% methanol extract of *A. annua* and ethanol extract of *C. annuum* was more effective on most biological parameters of *P. gossypiella*. Also, *A. annua* has lower efficacy than *C. annuum*. The use of *A. annua* and *C. annuum* can be recommended against *P. gossypiella*. (Reda, 2014)

Effectiveness evidence of *C. annuum* as an insecticide was obtained by direct extraction after putting chili pepper with distilled water in a blender. This mixture was diluted in water, filtered and applied to corn crops against armyworm *Spodoptera frugiperda*, showing that treatment with this plant acqueous extract can control pest through constant fumigation. (Navarro, 2013)

The ability to control tomato moth *Tuta absoluta* under greenhouse conditions was also evaluated. Chili pepper extract as insecticide mixed with soap solution is effective for controlling larvae states especially 24 hours after treatment. (Trabuco, 2015)

3.4.3 CLOVE, *Syzygium aromaticum* L.

Dried flower buds of *Syzygium aromaticum* L. synonymous. *Eugenia caryophyllus* S., are recognized worldwide as clove, having resemblance to a smithy nail. In the ecuadorian kitchen it is used for traditional food cooking according to the study published in the book “Plantas Útiles del Ecuador”. (Rios, 2007)

This flower bud essential oil is mainly propenylphenol also known as eugenol, existing in free and eugenyl acetate form; it also contains several terpene compounds: aliphatic, aromatic and heterocyclic, with the presence of at least 10% β-caryophyllene. (Bruneton, 2001)

3.4.3.1 STUDIES ON NATURAL EXTRACTS OF SYZYGIUM AROMATICUM

Anti-cholinesterase activity determinations with methanolic extracts of *S. aromaticum* essential oil, showed that eugenol has better inhibitory activity on the enzyme than extract and oil. (Dalai, 2014)

Antimicrobial and antifungal activity of three plants essential oils: *Syzigium aromaticum* (Clove), *Nigella sativa* (Kalonji) and *Eruca sativa* Miller (Taramira) were tested by disk diffusion assay. With clove essential oil Gram – bacteria were more sensitive compared to Gram +, while *T. mentagrophyte* presented good sensitivity among fungi tested group. (Shoaib, 2014)

Bioactivity of five essential oils was tested for toxicity and repellent activity: clove, mustard, cinnamon, castor and eucalyptus; at three different concentrations, against *Bruchidius incarnatus* adults or bean beetle. Clove was the second oil showing better toxic activity, but poor repellent activity. (Fouad, 2013)

Essential oils of *Syzygium aromaticum* inflorescences and leaves were tested for ovicidal and larvicidal activity against *Aedes albopictus*. Interference in eggs hatching is shown. Leaf oil was more toxic than inflorescence oil. (Sumangala K., 2009)

Antimicrobial activity of clove essential oil against *Vibrio spp.*, *Edwardsiella spp.*, *Aeromonas spp.*, *Escherichia coli*, *Flavobacterium spp.*, *Salmonella spp.* isolated from aquaculture, and seven reference bacteria, *Escherichia coli* (ATCC 25922), *Citrobacter freundii* (ATCC 8090), *Aeromonas hydrophila* (ATCC 49140), *Pseudomonas aeruginosa* (ATCC 35032), *Streptococcus agalactiae* (ATCC13813), *Edwardsiella tarda* (ATCC 15947) and *Yersinia enterocolitica* (ATCC 23715); showed huge potential to substitute commercial antibiotics. (Lee, 2009)

Antifungal activity of four plants hydroalcoholic extracts, cinnamon (*Cinnamomum verum* Presl.), anise (*Pimpinella anisum* L.), black seed (*Nigella sativa* L.) and clove (*Syzygium aromaticum* Merr L. & Perry.), was determined against the fungus *Rhizoctonia solani* that attacks *Pisum sativum* root. The results showed high inhibitory power on the fungus at 1% clove concentration. Also in greenhouse tests antifungal activity is confirmed, an increase in the plants proportion that survive and decrease in disease incidence is observed. (Abdulaziz A. Al-Askar, 2010)

Evaluation of antibacterial activity of decoction and infusion extracts, single or in combination, of *Syzygium aromaticum*, *Laurus nobilis* and *Cuminum cyminum* plants was performed against five bacterial strains: *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis* and *Proteus vulgaris*. It was shown that combined infusions of the three plants have increased activity against *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa*, better than pure ones. (Chaitanya Sravanthi Kota, 2013)

Antifungal capacity of clove essential oil was tested against plant and animal pathogens: *Fusarium moniliforme* NCIM 1100, *Fusarium oxysporum* MTCC 284, *Aspergillus sp.*, *Mucor sp.*, *Trichophyton rubrum* and *Microsporum gypseum*. All species were inhibited in plate diffusion tests, it is concluded that eugenol contained in clove essential oil is responsible for the action. (Inder, 2011)

Toxicity test of clove oil against four fungi strains: *Alternaria*, *Fusarium chlamydosporum*, *Helminthosporum oryzae* and *Rhizoctonia bataticola* by the plate diffusion method, demonstrated fungicide capacity of clove oil against phytopathogenic fungi. (Beg & Ahmad, 2002)

Insecticidal activity of essential oils from five species: *Citrus sinensis* L. Osbeck, *Eucalyptus camaldulensis* Dehnh, *Laurus nobilis* L., *Lavandula officinalis* L.,

Syzygium aromaticum L., were evaluated against *Sitophilus oryzae* rice weevil L., and the red flour beetle *Tribolium castaneum*. Treatments of *L. officinalis*, and *S. aromaticum* had the best performance. (El-Bakry, 2016)

Essential oils of lemon peel (*Citrus limon*), orange peel (*Citrus aurantium*), cinnamon (*Cinnamomum zeylanicum*), clove (*Syzygium aromaticum*), red currant (*Ribes rubrum*), Elite variety, black currant (*Ribes nigrum*) Tinker variety were evaluated by disk diffusion tests against *Bacillus subtilis* ATCC 6633. The best results were obtained with cinnamon oil and clove oil. (Butu, 2013)

3.4.4 ARTEMISIA, *Artemisa vulgaris*

Mugwort leaves are known in Ecuador as " Altamisa or Marco ". It has been traditionally used as an antirheumatic when leaves are cooked on fire and applied to the affected area. Leaves decoction is used for evil air and the frights. It eliminates fleas and insects, relieves hemorrhoids pain acting as an analgesic, and prevents the formation of internal abscesses. It is also used in ritual baths. (Rios, 2007)

It contains mainly monoterpene fractions, sabinene, myrcene, cineole, camphor, borneol. The sesquiterpene fraction dominates in the mugwort where caryophyllene oxide, β-cubebene and β-elemene (6%) are the major constituents. (Judzentiene & Buzelyte, 2006)

3.4.4.1 STUDIES ON NATURAL EXTRACTS OF ARTEMISA VULGARIS

Studies for the development of a potential acaricide were carried out with different parts of 12 plant species, using its methanol extracts against the mite *Tetranychus urticae* Koch. *Lolium perenne* extracts, *Artemisia vulgaris* (leaves), *Chenopodium album* (Leaf and flower), and *M. azedarach* L. (fruit) had significant acaricidal effects. (Yanar, 2011)

Essential oils of nine plant species were proved against adult wheat weevil (Coleoptera: Curculionidae), essential oil of *A. vulgaris* had very low or no effect. (Kordali, 2012)

Studies of volatile compounds in *Artemisia vulgaris* leaves found the presence of terpenes such as camphor, eucalyptol, α-pinene, β-pinene. Commercially available

compounds were tested for phytotoxicity. Bioassays revealed that apparently there is a synergistic action with other unproven compounds that give this plant an allelopathy quality. (Barney, 2005)

Lipophilic phenolic extracts of *Artemisia* various species were tested to prove their antimicrobial activity against: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 6633, *Proteus vulgaris* ATCC 4636, *Candida albicans* ATCC 885-663, using the disk diffusion method. Various activity degrees were found except the test for *C. albicans* that showed no sensitivity to the extracts. (Kovalyova, 2013)

3.4.5 HORSETAIL, *Equisetum arvense*

This plant infusion known as “horsetail” has been used to treat kidneys inflammation, as a diuretic, and also to cure the liver. Other traditional medicines are brittle fingernails, hair loss and rheumatic diseases treatment. *E. arvense* has recently shown to have antibacterial, antifungal, antioxidant, analgesic, anti-inflammatory, antidiabetic, antitumor, cytotoxic and anticonvulsant activities. (Asgarpanah & Roohi, 2012)

Gas chromatography analysis revealed the presence of six major substances: tetramethyl hexadecenol, hexadecanol, octadecanoic acid methyl esther, phthalic acid and gibberellic acid. (Yilmaz, 2014) Other components are flavonoids, caffeic acid ester, silicic acid, and pyridine alkaloids. (Commission, Pilot project: Proposal for approbation of basic substances, in the context of Regulation, 2013)

3.4.5.1 STUDIES ON NATURAL EXTRACTS OF EQUISETUM ARVENSE

Further investigations to determine antimicrobial activity of plant extracts including *Equisetum arvense* against *E. coli* strains isolated from cow's milk showed sensitivity to the plant extract. (Hleba, 2013)

Methanol extracts of *Equisetum arvense* were tested against Gram negative bacteria: *E. coli*, *Listeria*, *Pseudomonas*, *Serratia*; and Gram positive bacteria: *Enterococcus*, *Lactobacillus*, *Paenobacillus*, *Brochothrix*, *Staphylococcus*, by disk diffusion method, showing different degrees of antibacterial activity. (Vatlak, 2014)

The European Commission conducted a pilot project for *Equisetum arvense* basic substance application in plant protection on grapevines and apple trees to control foliar fungi diseases and also as a plant strengthener. (Commission, Pilot Project: Proposal for approbation of basic substances, in the context of Regulation (EC) No. 1107/2009, 2013) The European Food Safety Authority (EFSA) concluded that it does not meet the criteria as a basic substance, there are no relevant evaluations in the European Union either as a food or as a medicine. The decoction composition is unclear, although the presence of alkaloids, phytosterols, tannin, triterpenoids, flavonoids and phenolics is claimed. A qualitative and quantitative consumer risk assessment was not possible. (EFSA, 2013)

3.5 EXTRACTS PREPARATION DESCRIPTION

Plant material amount to be used for extracts preparation was determined in accordance with several manuals about preparations with native plants that can be applied to crops with the aim of fighting pests that infect them. (ECOTENDA) (FAO, Los Biopreparados para la Produccion de Hortalizas en la Agricultura Urbana y Periurbana, 2013) (Funsalprodese, 2000) (Hernandez, 2000) (ProDeSoC-IPADE) (Quiros, 2004)

Extracts of *Equisetum arvense*, *Capsicum annum* and *Ruta graveolens* were prepared in aqueous solutions, whereas *Syzygium aromaticum* was prepared with alcoholic extraction. The objective was to determine if plant extracts prepared with simple and affordable methods will be effective to induce defense mechanisms and fight pests when applied on crops, considering that farmers do not have appropriate technology for active substances extraction.

3.5.1 *Equisetum arvense*

Decoction of 5 g freshly harvested and finely chopped branches with 100 mL of milliQ water, macerated for 24 hours protected from light. It was filtered through filter paper and the liquid was brought to pH 6.5.

3.5.2 *Capsicum annum*

25 g of finely chopped fresh “Rocoto” chili peppers in 100 mL milliQ water, macerated for 24 hours protected from light. It was filtered through filter paper and the liquid was brought to pH 6.5.

3.5.3 *Ruta graveolens*

12 g of fresh rue leaves with flowers and without thick stems are left to macerate in 100 mL of milliQ water protected from direct light for 24 hours. It was filtered through filter paper and the liquid was brought to pH 6.5.

3.5.4 *Syzygium aromaticum*

Cloves were finely ground, 20 g of powder were placed in 100 mL of 70% alcohol, macerated for 72 hours in a dark place, then it was filtered through filter paper and concentrated by evaporation. Subsequently 1mL of this solution is mixed with 100 mL of water and the liquid was brought to pH 6.5.

3.5.5 *Artemisia vulgaris*

5 g of fresh mugwort leaves are left to macerate in 100 mL of milliQ water protected from direct light for 24 hours. It was filtered through filter paper and the liquid was brought to pH 6.5.

3.5.6 Sodium hypochlorite

A solution of 250 mg/L in milliQ water was prepared from a commercial product purchased in local markets with a concentration of 5% w/v. The pH was adjusted to 6.5.

3.5.7 Sodium deoxycholate

A solution of 200 µM prepared from a commercial lyophilized product was prepared in milliQ water, the pH was adjusted to 6.5.

CHAPTER 4

EXPERIMENT DEVELOPMENT

CHAPTER 4

4. EXPERIMENT DEVELOPMENT

4.1 MATERIALS AND METHODS

4.1.1 OBTAINING PLANT MATERIAL

Seedbeds were prepared from certified tomato seeds that were transplanted and seeded in a humus natural substrate; the state of seedling was reached when they were about 60 days and had an average height of 15 centimeters. Figure 21. Plants were maintained in a growth chamber at constant temperature of 25 ^{o}C, illumination was kept for 16 hours and moisture controlled by regular watering. Figure 22.

Figure 21. Tomato seedlings and transplantation in pots with humus. Source: the author.

Figure 22. Tomato plants in the growing chamber ready for the treatments. Source: the author.

Once the plants were treated, pots were marked and returned to the growth chamber. Figure 23.

Figure 23. Treatment identification system. Source: the author.

4.1.2 Substances applied to the plants

Water-based substances were applied by spraying on tomato plants, following a completely randomized experimental model with three levels and three replicates.

4.1.2.1 First group of Treatments:

Each plant extract was prepared by boiling or macerating a known amount of the plants *Equisetum arvense* and *Artemisa vulgaris*. The extracts were then diluted 1:3 ratio before spraying. Chlorine at a final concentration of 250 mg/L in water. Sodium deoxycholate diluted at a final concentration of 200 µM in water. Myclobutanil was diluted at the recommended dose of 125 g/L. All the solutions were adjusted to pH 6.5.

APPLIED TREATMENTS, first group.

TREATMENT No. 1
Cooking of the plant *Equisetum arvense* (cola de caballo), diluted in water.

TREATMENT No. 2
Maceration of the plant *Artemisa vulgaris* (altamisa), diluted in water.

TREATMENT No. 3
Comercial sodium hypochlorite diluted at a final concentration of 250 mg/L in water.

TREATMENT No. 4
Comercial sodium deoxycholate diluted at a final concentration of 200 μM in water.

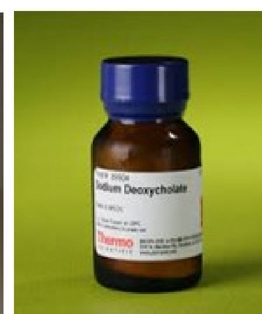
TREATMENT No. 5
Comercial fungicide diluted in wáter at the recommended dose 0,2 g/L.

Figure 24. First group of applied treatments. Source: the author.

4.1.2.2 Second group of Treatments

A known specify amount of *Capsicum annum* fruit was cut and submerged in water for 24 hours, leaves of the plant *Artemisa vulgaris* were boiled in water and subsequently diluted 1:3 before spraying. Chlorine at a final concentration of 250 mg/L in water. Sodium deoxycholate diluted at a final concentration of 200 μM in water. All the solutions were adjusted to pH 6.5.

APPLIED TREATMENTS, second group.



TREATMENT No. 1
Maceration of the plant *Capsicum annuum* (aji), diluted in water.

TREATMENT No. 2
Maceration of the plant *Artemisa vulgaris* (altamisa), diluted in water.

TREATMENT No. 3
Comercial sodium hypochlorite diluted at a final concentration of 250 mg/L in water.

TREATMENT No. 4
Comercial sodium deoxycholate diluted at a final concentration of 200 μM in water.

Figure 25. Second group of applied treatments. Source: the author.

4.1.2.3 Third group of Treatments

Chlorine at a final concentration of 250 mg/L in water. Sodium deoxycholate diluted at a final concentration of 200 µM in water. All the solutions were adjusted to pH 6.5. Rue and pepper solutions were used in an undiluted form.

Figure 26. Third group of applied treatments. Source: the author.

4.1.3 RNA Extraction and quantification

For RNA extraction Plant Total RNA Kit from Sigma was used. Leaves were harvested according to each plant treatment and ground with the addition of liquid nitrogen using a mortar and a pestle. Approximately 100 mg of a pool of leaves were weighted in 1,5 mL conical tubes. Total RNA was extracted according to manufacturer's instructions.

The typical yield is 20-60 µg depending on both the type of tissue and the state of development. To remove genomic DNA, which may be coeluted with RNA, RNA was treated with DNase I (Invitrogen) before the quantitative PCR. (Invitrogen).

RNA concentration was measured in an Invitrogen Qubit Fluorometer 2.0. (Qubit).

4.1.4 cDNA Synthesis

For cDNA synthesis the Universal Transcriber cDNA Master Kit (Roche) was used. The kit provides components necessary for cDNA synthesis including hexamer primers, nucleotides, buffers and enzymes.

RNA obtained from the previous step was used as template in the reverse transcription reaction, Table 4. (Roche, Transcriptor Universal cDNA Master User Manual).

Table 4. Reverse transcription reaction protocol.

COMPONENT	VOLUME	FINAL CONCENTRATION
PCR grade water	X µl	-
Universal Transcriptor Buffer	4 µl	1X
Reverse Transcriptase Universal Transcriptor	1 µl	1X
RNA template	X µl	2.5 µg (up to 1 µg)

Thermal cycler was programmed according to the data indicated in Table 5.

Table 5. PCR thermal cycler Protocol

STEPS	ACTION
Hybridization	+25 ^{o}C for 5 minutes
Reverse transcription	+55 ^{o}C for 10 minutes
Denaturation	+85 ^{o}C for 5 minutes
Hold	+4 ^{o}C (∞)

4.1.5 Quantitative PCR Reaction

For the Quantitative PCR reaction, Figure 27, FastStart Essential DNA Green Master Kit (Roche) was used, with the equipment Roche Lightcycler Nano Real Time PCR System.

Data is analyzed in the linear amplification phase (linear logarithmic phase) by means of relative quantification comparing the expression levels between the gene of interest and reference genes. (Roche, Technical Note, 2005)

The system uses SYBR green dye, which intercalates between DNA and fluoresces once bound to DNA. The amount of pigment incorporated is proportional to the amount of amplified DNA. The dye emits fluorescence at 520 nm.

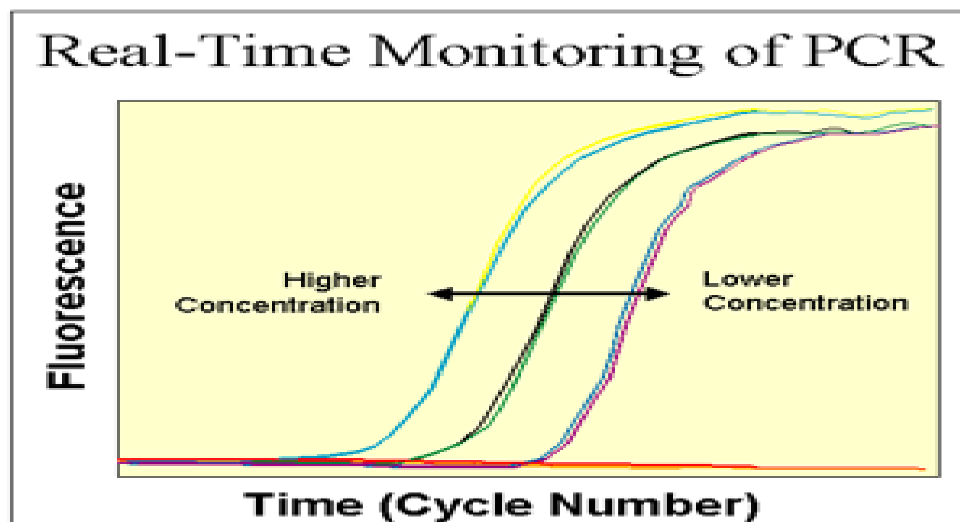


Figure 27. Quantitative PCR. Source: https://dna.utah.edu.

Melting curve analysis was performed to prove the reaction specificity, the test is considered valid if it produces a single and well defined peak. (Eurogentec) The expression levels of PR-1b1, PR-1a2, SlWRKY 3, SlWRKY 23, SlWRKY 39, and MAPK genes were investigated using as reference actin AC and elongation factor EF genes, whose selection was based on their expression stability. Figure 28.

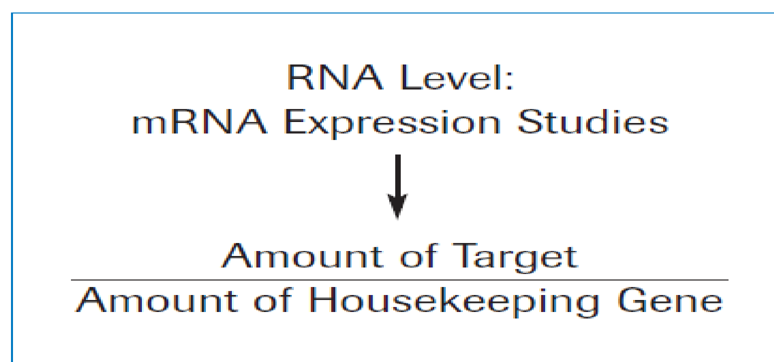


Figure 28. Roche Applied Science Technical Note No. LC 13/2001

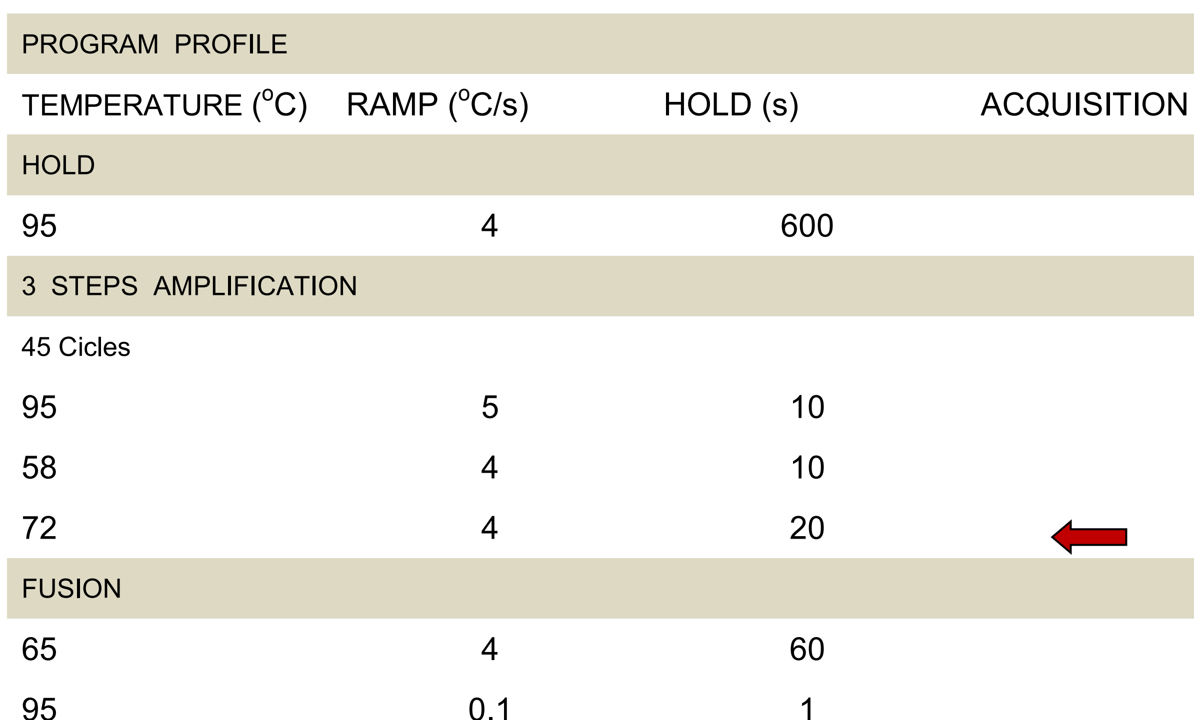
According to the protocol used for qPCR reaction, FastStart Universal SYBR Green Master (Roche), Table 6 details the components used in the reaction.

Table 6. Components for qPCR reaction.

COMPONENT	VOLUME
PCR grade water	4.5 µl
PCR primer (10 pmol/µl)	1 µl
Master Mix 2X conc.	7.5 µl
cDNA (diluted 1:10)	2 µl
Total Volume	15 µl

In order to obtain results that can be statistically analyzed, two PCR reactions were performed for each sample obtained from different treatments, also for housekeeping genes used and positive/negative controls. Table 7 shows the protocol used to program Roche Lightcycler Nano which was used for quantitative PCR (qPCR) amplification.

Table 7. Protocol for qPCR amplification.



PROGRAM PROFILE			
TEMPERATURE (^{o}C)	RAMP ($^{o}C/s$)	HOLD (s)	ACQUISITION
HOLD			
95	4	600	
3 STEPS AMPLIFICATION			
45 Cicles			
95	5	10	
58	4	10	
72	4	20	⬅
FUSION			
65	4	60	
95	0.1	1	

Primers used for target genes amplification correspond to defense genes, transcription factors and regulatory proteins in plants. For primers sequence, size, and suitable melting temperature (Tm), a bioinformatic analysis was performed using the tools of the National Center for Biotechnology Information (NCBI) and Plant Transcription Factor Database (Plant TFDB).

Sequences of primers used for quantitative PCR (qPCR) are shown in Table 8.

Table 8. Primers used for qPCR.

PRIMER	SEQUENCE 5' – 3'
PR1a2 Forward	AAT TGT GGG TGT CGG AAA AG
PR1a2 Reverse	AAG ACG TTG TCC GAC CCA AT
PR1b1 Forward	AAT TGT GGG TGT CCG AGA GG
PR1b1 Reverse	TAA GGA CGT TGT CCG ATC CAG
ACTIN Forward	CAC CGA GAG AGG TTA CAT GTT CA
ACTIN Reverse	AAC CTC TCA GCA CCA ATG GTA A
EFNT Forward	ACA AGA TGG ATG CTA CCA CCC
EFNT Reverse	GGT CCC TTG TAC CAG TCG AG
SlWRKY8 Forward	TAA TTC TGC CGG AAA GCC TC
SlWRKY8 Reverse	ATG CTT ATT GCC GGT ACT CGA
SlWRKY23 Forward	TGG AGA TGC TGA TGG GGA AG
SlWRKY23 Reverse	AAA CGC AAA TCT CGG CTC TT
SlWRKY39 Forward	GCG GTA ATG CCA AGA CAA AC
SlWRKY39 Reverse	TCA GTT CCT GGT GAT TTA CGC
MAPK3 Forward	GCA AAT TGC GAC CTC AAG AAT GG
MAPK3 Reverse	AGC TCA GTA ATG AGT CTC AGC TG
MAPK6 Forward	GTG ATT TTG GGC TAG CTC GTG
MAPK6 Reverse	CCA TAA GCA GAC GTA GTC GGT GC

For accurate gene quantitation (qPCR) of studied genes, Genorm analysis of Vandesompele et al. was performed. It allows the expression normalization of data

taking into consideration the geometric mean of housekeeping genes, in this case Actin and Elongation Factor. (Vandesompele & al., 2002)

L.esculentum PR1a2 gene

GenBank: Y08844.1

GenBank Graphics

```
>Y08844.1 L.esculentum PR1a2 gene
AGTTGGACATAGATTCTTTATTTCTTTAGAATTATAAAATATACGTATTCAAAATTTAAGTGGTCGTAAA
TTAATACAAAAGTCAAGAAAACTCTTCATTCAAAATGGAATATGAATTCACGCATCAGTCTTTGAACACT
TTAACACTTTAATTGAAAAATTATGTACAATATATCCGTTCAAAACTACTTTATGCGCATATATATATTG
ATTAACCATATCAATTTTTTTATTCTCTACTTATTACCTTATGTAAACACCTAACTAATTATTCATAAAT
TCAACTTCAACTCTTCTTAAACGTTTTAAAAATTAATAATTCCCATTTGAAGCAGCCATGATGTGAGGCA
GTTAATTTCTACACAAAGAACCACATATTAAGTGTCCACACTTTGTTTATTATGTTTTCATTATATCCAA
ATTGCTTAATTTCAATGACTAACAAAAGAATAATTTTCTTTTCATACATTCAAGGCGGCTCAATAATATT
GATGACCCAAAATCAATTTGTCATAAAATATTTTATAGGTAAAAACTGAATTGCATCCTTTTTCTATTAG
AGGTTTTGAATACAAACCCCGAAACATAGATAAATATTATTTAGATTGAATTTTCCTTTAAAAAAGCTTA
CGGTGATGCAAATTAAATTAATTAAGACTCCAAAACACATTTCGAATATCTAAATTAAAGTATGACAATT
ATTTAAAGTATATATAGTAATCCCTAAATCACACGATATGGAAGGTATTGATCACATTTGAATTTCTTCA
CATTATTAAATTCACATGTTTGAATAATACAATCAAAGTTAGATATAAAGAAAAAAAAATATTCATTTTT
TGAAAAAACTTAAAAAGGACCAAAAGTAATTAATTCTAAAAAGTTTAAGGACCAAAATTGCTTAGCAAAA
TTCCACAATTTTTACTTATAAATACACTACTTATCTCACATTTATAATCACAACAACTTAAATTTATTTT
CTCTCAAAGCAAAAATGGGGTTGTTTAACATGTCATTGTTACTTATGACTTGTCTCATGGTATTAGCCAT
ATTTCACTCTTGTGATGCTCAAAATTCACCCCAAGACTATCTTGAGGTTCACAACGACGCCCGTGCCCAA
GTCGGAGTCGGGCCAATGTCTTGGGATGCCGACTTGGAATCCCGAGCACAAAGCTATGCCAACTCAAGAG
CGGGTGATTGTAACTTGATTCATTCTGGTTCAGGGGAGAATCTTGCCAAGGGTGGTGGTGACTTCACGGG
GAGGGCCGCTGTGGAATTGTGGGTGTCGGAAAAGCCAAACTACAACTACGATACGAATGAATGTGTTAGC
GGAAAAATGTGCGGACATTATACTCAAGTAGTCTGGCGTGACTCAGTTCGACTAGGTTGTGGTCGGGCTC
TTTGCAACGACGGGTGGTTTATTTCTTGCAACTATGATCCTGTAGGCAATTGGGTCGGACAACGTCTTAC
TAAAATGTTTTTTTTTTTTGTATGATGTGTAAGGATCAAATAATTATTATTATTTCCTTTGATCTTTGCT
AGTATGAATAATTCCACATACCATATGTTCATGGTATAGTGGGCTTAAGTTGATAATAAATAAAGTTTTT
CTATTTTATTAAATAAGATTAAAATACATGGCATAATGGTAAAATAATTATTATTGTCTTATGCAAATTA
CGAGAGTTAAATTATTAATTTTTTTAAGTGTAAAGACGATCATAGATTCTTTATATCTTATAAAATGAAT
TATATATTTAAAAACGGTTTGAAATGATATATAAGTAGAGGCGGATCTAGGATTTGAAGGTAATGGGTGT
CATATCGTTAAGTCTCAATCGTTTGTGATTTTTCATCACATATGCAATATTATAATAATGTTTATTAAAG
TCAAACGTAGTCCATGACTTAATGGTATTAACATTGTCTTGTCATGTTTGGATTAGCGTTTGAGTCGAAG
CGTCAACATTATTATTTGCAAAAGAAATGGAAAATGCATTAAAATGAGCAATATGAGAATCGAACGACGC
CATGTGCCTACTTGTGTCCAGGGGTGACAGATTAGTTTTTCATAAGTTTTATATGCATAAATATATACAT
ACACATGTATACATAAAATTTCAACCAAGATCATTGGATGCCGTGACACCCTACCATGATACATATATCC
GCCCCTGCACATATTAGTACATGTGAGTCTG
```

Figure 29. PR1a2 gene FASTA sequence. Source National Center for Biotechnology Information NCBI.

Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	AATTGTGGGTGTCGGAAAAG	Plus	20	1275	1294	56.53	45.00	4.00	0.00
Reverse primer	AAGACGTTGTCCGACCCAAT	Minus	20	1468	1449	59.60	50.00	5.00	2.00
Product length	194								

Products on potentially unintended templates

>NM_001321040.1 Solanum lycopersicum pathogenesis-related protein (PR1a2), mRNA

```
product length = 197
Forward primer  1    AATTGTGGGTGTCGGAAAAG  20
Template        302  ....................  321

Reverse primer  1    AAGACGTTGTCCGACCCAAT  20
Template        498  .G..................  479
```

Figure 30. PR1a2 primer pair analysis. Source National Center for Biothechnology NCBI.

Solanum lycopersicum PR protein (PR1b1), mRNA

NCBI Reference Sequence: NM_001247385.2

GenBank   Graphics

```
>NM_001247385.2 Solanum lycopersicum PR protein (PR1b1), mRNA
ATCACAATAACTTAGATTTATTTTCTCTCCACTAAACCTAAAGAAAAATGGGGTTGTTCAACATCTCATT
GTTACTCACTTGTCTCATGGTATTAGCCATATTTCACTCTTGTGAGGCCCAAAATTCACCCCAAGACTAT
CTTGCGGTTCATAACGATGCCCGTGCCCAAGTCGGAGTCGGGCCTATGTCTTGGGATGCCAACTTGGCAT
CCCGAGCACAAAACTATGCCAACTCAAGAGCTGGTGATTGTAACTTGATTCATTCTGGTGCTGGGGAGAA
TCTTGCCAAGGGTGGTGGTGACTTCACGGGGAGGGCAGCCGTGCAATTGTGGGTGTCCGAGAGGCCAAGC
TATAACTACGCTACCAACCAATGTGTTGGTGGAAAAAAGTGTAGACATTATACTCAAGTAGTCTGGCGCA
ACTCAGTCCGACTAGGTTGTGGTCGGGCACGTTGCAACAACGGATGGTGGTTCATTTCTTGCAACTATGA
TCCTGTAGGCAACTGGATCGGACAACGTCCTTACTAAAATGATGTATACTTATGACATGTTGCTAGTATT
AAATAAAATTCTCATATGAGACGTCGAGAAGTTAAAATTTAAGTTTGACATATGAATCAAGTCAAACTCC
TATCTAAAATATTAAGGGATTAAATATTGAACATCTATAATTATTATTATTTCCCTTTTGATGTTGCTAA
TATGAATAATTCCACATACCATATGTTCATAATGGGCTTAAGTTGATTATTAAGTACTGCATCTTCTTGT
TTCCATAAAACATTAATATACATAAAATTTTAATTAA
```

Figure 31. PR1b1mRNA FASTA sequence. Source National Center for Biotechnology Information NCBI.

Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	AATTGTGGGTGTCCGAGAGG	Plus	20	325	344	59.68	55.00	4.00	0.00
Reverse primer	TAAGGACGTTGTCCGATCCAG	Minus	21	523	503	59.52	52.38	5.00	1.00
Product length	199								

Products on intended target

>NM_001247385.2 Solanum lycopersicum PR protein (PR1b1), mRNA

```
product length = 199
Forward primer  1    AATTGTGGGTGTCCGAGAGG  20
Template        325  ....................  344

Reverse primer  1    TAAGGACGTTGTCCGATCCAG  21
Template        523  .....................  503
```

Figure 32. PR1b1 primer pair analysis. Source National Center for Biothechnology NCBI.

```
>Solyc02g093050.2.1
ATGGCTGTGGAGCTAATGATGGATTACAGAAGCACTAGAAATAACAATAATACTAATTGT
ATTAACTTCGTTGCGAAATTAGAAGAAAAATCAGTTGTGCAAGAAGCTGCTTCTGGTCTT
GAGAGTGTTGAGAAACTCATCAGATTATTATCACAGTCTCAATCTCAACAAATTCAGCAG
CAAAATAAGTCTCCAATGGAGATTGAAATGGTGGCTGATGCAGCTGTTACAAAGTTTAAG
AAGGTAATTTCACTTCTAGATCGAAACAGAACTGGTCATGCTAGATTCAGAAGAGCTCCT
TTGGCTAATAATAATTCTCCTTTGCCTTCAAATTCTAGTAAAGATTTTGTGGATACAAAA
GTGTATTCTCCAACTCCGATCCAACAAGTTCCTTTAATTACCTATGACCATTTTAACCCT
CTGGTTCCAAAGACGATAAGTTTCTCCTATTCACCGGAAATGTCTCGCACAAATTCCTTC
AATATCTCGTCGTTAACAGGGGAAACAGAGAGCAAACAACATTCTTCATCTAATTCAGCT
TTCCAGATGACCAATCTTTCTTCTCAAGTCTCTAATTCTGCCGGAAAGCCTCCATTGTCT
TCTTCTTCACTGAAACGAAAGTGCAGTTTATCGGAAAATGCCGTATCTGGAAAGTGCAGT
GGATCTTCCGGCCGATGCCATTGTTCCAAGAGAAGAAAGTTAAGACTAAAGAGGGTAGTT
CGAGTACCGGCAATAAGCATGAAACTGTCAGATATCCCACCGGATGATTACTCATGGAGA
AAATATGGACAGAAGCCAATCAAAGGATCTCCACATCCAAGGGGATATTACAAGTGTAGT
AGTGTAAGAGGGTGTCCAGCACGTAAACATGTTGAAAGAGCATTGGATGATCCAACTATG
CTGATTGTTACCTATGAAGGAGAACATAATCATTCACTTTCTGTTGCTGAAACAAGTAGT
CTCATTTTAGAGTCTTCTTAA
```

Figure 33. SlWRKY7 sequence. Source Plant Transcription Factor Database PlantTFDB.

Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TAATTCTGCCGGAAAGCCTC	Plus	20	573	592	57.96	50.00	5.00	1.00
Reverse primer	ATGCTTATTGCCGGTACTCGA	Minus	21	740	720	59.59	47.62	4.00	4.00
Product length	168								

Products on potentially unintended templates

>NM_001346926.1 Solanum lycopersicum WRKY transcription factor IId-4 (WRKYIId-4), transcript variant 2, mRNA

```
product length = 168
Forward primer  1    TAATTCTGCCGGAAAGCCTC  20
Template        774  ....................  793

Reverse primer  1    ATGCTTATTGCCGGTACTCGA  21
Template        941  .....................  921
```

>NM_001346925.1 Solanum lycopersicum WRKY transcription factor IId-4 (WRKYIId-4), transcript variant 1, mRNA

```
product length = 168
Forward primer  1    TAATTCTGCCGGAAAGCCTC  20
Template        867  ....................  886

Reverse primer  1     ATGCTTATTGCCGGTACTCGA  21
Template        1034  .....................  1014
```

Figure 34. SlWRKY7 primer pair analysis. Source Plant Transcription Factor Database PlantTFDB.

```
>Solyc01g079260.2.1
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AACGGTTTTGGAGTTACTAATAACCACTCATTCATCTCAGATTACAGCATTAACCCATCA
TCTCTGGGTTTCATGGAGTTATTGGGTTTTCATCAAGACTTTTGTTCAGTTTTTGAGTTA
CCTAAAGAAGAAAATCACTATCCTGCTGTTTGTGTATCTGAAGAAGAACTAAAGCCGCCA
TCATCATCATCTGTAGCAGCAGCTGAGAAACAAAAAAGTAGTACTACTACTGTAGTTGCT
ACAGGTAACGTATTGAATACGCCATCTACCCCTAATTGCTCCTCCATTTCCTCTGAAGGA
CATGGAGATGCTGATGGGGAAGTAGAAAATCATGACCAAAAAAACACAAACGCTAAACAA
CAGTTGAAAGCGAAGAAAACAGTGAGTCAGAAGAAACAGAAAGAGCCGAGATTTGCGTTT
ATGACAAAAAGTGAGGTTGATTTTCTTGAAGATGGTTATAGATGGAGAAAATACGGTCAA
AAAGCTGTCAAAAACAGTCCTTTCCCAAGGAACTATTATCGCTGCACAAATGCAACATGT
AACGTCAAGAAGAGAGTTGAGCGATGTTTCAGTGACCCAAGCATTGTGGTGACTACCTAC
GAAGGAAAACATACTCATCCAAGTCCCATGAATACGATGATCTCCCGTCCTAACTGCTAT
CCAATAAATCCAGTACTCCCTTCACTTGGAACCTACACTCTGCCAATGCAGTTCAACGCC
AATCAGTCCTTCAACGACAACTTAACGAGTTCTAATTTAGCCATCAATCATCAGCTTGAT
CATGCTGCTTTTGTTGCTCAAGGAAGGCGTTTTTGCAGTACTAACGAAATTCTGGAAGAC
CAGGAGAATGATCTACAGAATCTTATGCCTTCCGCGGTGCTAAAACATGACTACAACAGA
TGA
```

Figure 35. SlWRKY23 sequence. Source Plant Transcription Factor Database PlantTFDB.

Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TGGAGATGCTGATGGGGAAG	Plus	20	363	382	59.16	55.00	2.00	0.00
Reverse primer	AAACGCAAATCTCGGCTCTT	Minus	20	480	461	58.48	45.00	2.00	0.00
Product length	118								

Products on potentially unintended templates

>XM_004229329.2 PREDICTED: Solanum lycopersicum probable WRKY transcription factor 23 (LOC101263542), mRNA

```
product length = 118
Forward primer  1    TGGAGATGCTGATGGGGAAG  20
Template        440  ....................  459

Reverse primer  1    AAACGCAAATCTCGGCTCTT  20
Template        557  ....................  538
```

>XM_010316116.1 PREDICTED: Solanum lycopersicum dystroglycan-like (LOC104645097), mRNA

```
product length = 313
Forward primer  1   TGGAGATGCTGATGGGGAAG  20
Template        51  ..CGT..T..C.........  70

Forward primer  1    TGGAGATGCTGATGGGGAAG  20
Template        363  .T.GAG...........G..  344


product length = 241
Forward primer  1   TGGAGATGCTGATGGGGAAG  20
Template        51  ..CGT..T..C.........  70

Forward primer  1    TGGAGATGCTGATGGGGAAG  20
Template        291  .TT.AC..G...........  272
```

Figure 36. SlWRKY23 primer pair analysis. Source Plant Transcription Factor Database PlantTFDB.

```
>Solyc03g116890.2.1
ATGGAGTTCACAAGTTTAGTTGATACTTCTCTGGATTTGAACTTTAGACCTCTTCGAGTT
TCCGATGAATTACCAAAACAGGAAGTTGAGAGTAATTTCATAGGACTTGGAAGAGATCTG
GTACCTGTAAAAGATGAGGCAAGTAATTTAATAGAGGAACTAAATAGAGTAAATGCTGAA
AATAAGAAATTGACGGAGATGTTAACAGTTATGTGCCAGAATTACAATTCATTGAGGAAC
CAATTGACGGAATATATGAGCAAGCAGAATAGTAGTACTAGTGGAGCTGATCAGGATCAG
AACAGCGATGGATCGAAGAAAATTAAAATTGAAAACAACAACAATAATAATAATAATAAT
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TCATCTACAAAGAAACCAAGAGAAGAACACATTAAAACTAAGACTTCAAGAGTTTATATG
AGAACTGAACCATCTGATACTTCTCTTATAGTGAAAGATGGATACCAGTGGAGGAAATAT
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CCTACCTGTCCCGTTAAGAAAAAGGTTCAAAGAAGCGTGGAAGACCAATCGATTCTAGTA
GCAACCTATGAAGGAGAACACAACCATTCTAAAGTGGATACCGCAGGCCCTGTTACAACA
ACTTCCCCGTCTAGCCGATTTAACCCGAAAAATAATACTTATGCTGCTGCGGTAATGCCA
AGACAAACCTTAACTCTTGATTTGGCAGAACCAAAAACATTACAAAATGATATCAAAAAA
GTTCATAGCATTACAAGTACAAGTAGTGCAAGTGGTCAGAAGCGTAAATCACCAGGAACT
GATCAACAACAGCAAAATAGACCAGAGTTTCAACATTTCTTGATAGAACAAATGGCTTCA
TCATTGACTAAAGATCCAAGTTTTCAAGCAGCCTTAGCAGCCGCCATATCAGGAAAATTC
TTGCAAAATAATAGTAACACTAAGGATAAATAA
```

Figure 37. SlWRKY39 sequence. Source Plant Transcription Factor Database PlantTFDB.

Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GCGGTAATGCCAAGACAAAC	Plus	20	769	788	58.02	50.00	3.00	0.00
Reverse primer	TCAGTTCCTGGTGATTTACGC	Minus	21	902	882	58.30	47.62	3.00	2.00
Product length	134								

Products on potentially unintended templates

>NM_001316915.1 Solanum lycopersicum probable WRKY transcription factor 40 (LOC101246812), mRNA

```
product length = 134
Forward primer  1    GCGGTAATGCCAAGACAAAC  20
Template        846  ....................  865

Reverse primer  1    TCAGTTCCTGGTGATTTACGC  21
Template        979  .....................  959
```

Figure 38. SlWRKY39 primer pair analysis. Source Plant Transcription Factor Database PlantTFDB.

4.1.6 Amplicon size test with agarose gel electrophoresis

To verify the expected size of amplicons after ordinary PCR, an electrophoresis assay was run in 1,5 % agarose gel along with a 100 bp ladder, the results obtained fit with the expected size.

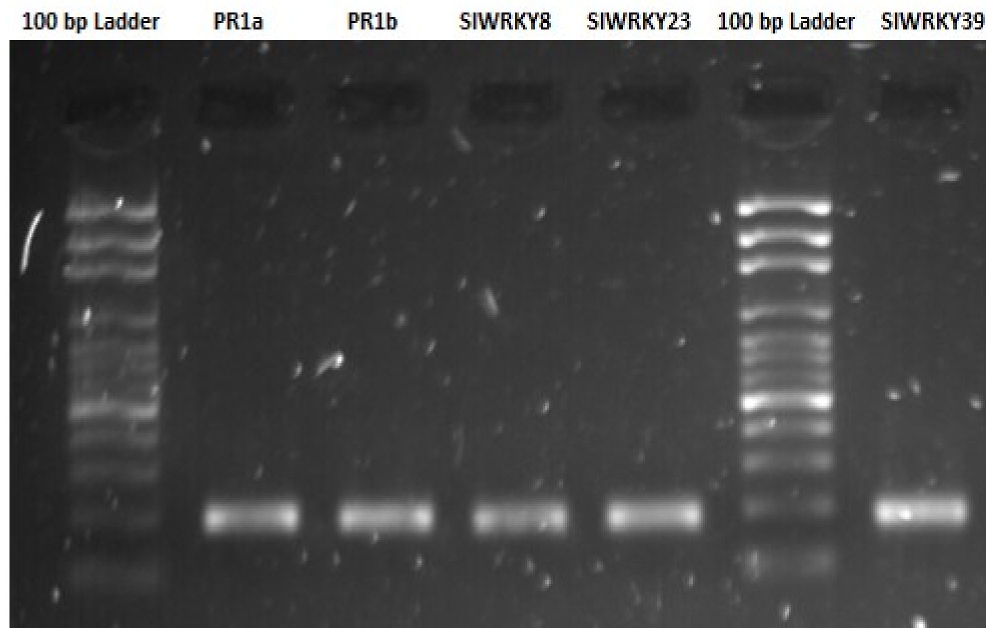


Figure 39. Amplified fragments of PR1a, PR1b, SlWRKY8, SlWRKY23, SlWRKY39, which are 200 bp long corresponding to the expected size compared with the 100 bp molecular ladder, 1,5 % agarose gel. Source: the author.

4.1.7 *In vivo* leaf infiltration Assay

Tomato and tobacco wild type leaves were infiltrated with different solutions according to Noda et al. (Noda & al., 2010) and Benouaret et al. (Bonouaret & al., 2014) and photographed 24 and 48 hours after treatment. The solutions used were fresh red chilli peppers (*Capsicum annum*, var. Rocoto), freshly-cut wild rue plants (*Ruta graveolens*) and clove powder (*Syzygium aromaticum*) purchased in local markets and used for plant extracts preparation. Aqueous extracts were prepared similarly to Sarpeleh et al. (2009). Briefly, pepper (25 g) and wild rue plants (12 g) where cut in small pieces and submerged in 100 mL of water for 16 to 24 hours in the darkness, filtered and used without dilution. Clove ethanolic extracts were prepared according to Pandey and Singh (2011) and used at a final concentration of 1% (v/v) in water.

Before plant treatments, the solutions were added with a wetting agent 30 mL/L (Agrotafix, containing polyethoxylates) and the pH titred to 6.5. Control treatments were performed with water alone (pH 6.5; negative control), diluted hypochlorous acid (250 mg/L, pH 6.5) and bile acid sodium deoxycholate (200µM, NaDC, pH 6.5) as positive controls. (Zarattini & al., The use of ECAS in plant protection: a green and efficient antimicrobial approach that primes selected defense genes, 2015) (Zarattini & al., The bile acid Deoxycholate Elicits defenses in Arabidopsis and reduces bacterial infection, 2016)

4.1.8 Histochemical GUS Assay

For this assay PR1a-GUS transgenic plants were used. Plants treated with different solutions ((Benzo [1,2,3] thiadiazole-7-carbothioic acid-S-methyl ester BTH, a salicylic acid analog, 140 mg/L was used as positive control) were assayed for GUS activity by histochemical assay carried out with a modified protocol. (Degrave & al., 2008) Fresh tobacco leaves were harvested 24 and 48 hours after the treatment and incubated in 50 mM Na_2HPO_4 and 10 mM EDTA, pH 7.0 containing X-gluc substrate (0.05% w/v).

Staining was performed in darkness at 37 °C for 16 to 20 hours. To remove chlorophyll, leaves were washed several times with hot 70% ethanol.

This is an *in situ* experiment, as long as GUS gene is present very rarely in plant tissues, Jefferson stated the possibilities existing for the localization of β-glucuronidase (GUS) in transgenic plants. (Jefferson, 1988) This is a versatile reporter of gene expression based on GUS enzymatic activity detection in tissues using an enzymatic stain.

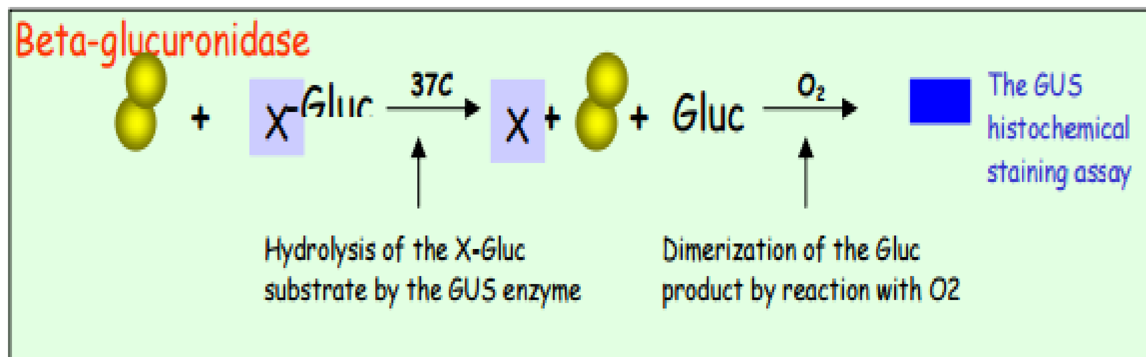


Figure 40. GUS Assay. Source: Plant Biotechnology Resource & Outreach Center, Michigan State University.

4.1.9 HPLC Salicylic Acid Assay

High Pressure Liquid Chromatography (HPLC), is a technique used to separate components in a mixture, consisting in a nonpolar stationary phase (column) and a mobile phase. The stationary phase is silica that has been treated with RMe_2SiCl. The mobile phase acts as a sample carrier.

The sample solution is injected into the mobile phase. The solution components migrate according to noncovalent interactions with the column. These chemical interactions determine the contents separation in the sample. The use of different detectors depends on compounds nature.

To evaluate the amount of salicylic acid in tomato leaves, the extraction procedure consisted in: leaf tissue was ground in liquid nitrogen using a mortar and pestle, 50 mg of powder was placed in a conic 1,5 mL tube with 1 mL of extraction mixture (10 % methanol, 1 % acetic acid, 89 % milliQ water), stirred by vortex for 30 seconds and put to degas in a Fisher sonicator for 5 minutes. Then the tubes were centrifuge at 13000g for 10 minutes, the supernatant was recovered in a fresh 1,5 mL conical tube. The extraction procedure was repeated once again with 500 µL of the extraction mixture and finally joined with the previous one. Before HPLC

analysis the samples were filtered with 0,45 µm Millex HV PVDF 33 mm syringe filters.

Quantification of salicylic acid SA was performed in a Waters 1525 HPLC with PDA detector, equipped with a XBridge C-18 column 4,6 x 150 mm at 36,5 ^{o}C. Mobile phases were 1:1 Acetonitrile-Water with Formic Acid 0,1 %, at isocratic mode, flow rate 0,6 mL/min. Detection wavelength 296 nm. The injection volume was 10 µL. Run time 4 minutes, salicylic peak appears at retention time 2,5 minutes.

4.1.10 Minimum Inhibitory Concentration MIC Assay

For this assay 100 µL of the extracts used to measure gene activation were filtered with 0,45 µm Millex HV PVDF 33 mm syringe filters, placed in a 96 well plate microtiter following the double dilution method with water. Powdery *Oidium* fungus spores were obtained from contaminated plants. 100 µl of spores, previously resuspended in PDB medium at a final concentration of $1x10^{8}$ spores measured in a Neubauer chamber,were added in each well. Chlorine 250 mg/mL was used as positive control and water as negative control. Table 9 shows the assay disposition. The experiment ran in duplicate. The plate was incubated at 23 ^{o}C for seven days. After each day 10 µL of Alamar Blue 10% was added to each column by careful pipetting in order to evaluate cell viability.

Table 9. Minimun Inhibitory Concentration MIC Assay, distribution of plant extracts, negative and positive controls.

<table>
<tr><th></th><th>1</th><th>2</th><th>3</th><th>4</th><th>5</th><th>6</th><th>7</th><th>8</th><th>9</th><th>10</th><th>11</th><th>12</th></tr>
<tr><td>A</td><td rowspan="8">Plant extracts, Oidim spore suspension and PDB.</td><td rowspan="8">Plant extracts, Oidim spore suspension and PDB.</td><td rowspan="8">Plant extracts, Oidim spore suspension and PDB.</td><td rowspan="8">Plant extracts, Oidim spore suspension and PDB.</td><td rowspan="8">Plant extracts, Oidim spore suspension and PDB.</td><td rowspan="8">Plant extracts, Oidim spore suspension and PDB.</td><td rowspan="8">Plant extracts, Oidim spore suspension and PDB.</td><td rowspan="8">BLANK</td><td rowspan="8" colspan="2">NEGATIVE CONTROL</td><td rowspan="8" colspan="2">POSITIVE CONTROL</td></tr>
<tr><td>B</td></tr>
<tr><td>C</td></tr>
<tr><td>D</td></tr>
<tr><td>E</td></tr>
<tr><td>F</td></tr>
<tr><td>G</td></tr>
<tr><td>H</td></tr>
</table>

4.1.11 Callose Determination Assay

For this assay an extraction procedure was carried out using 10 mg of powdered tomato leaves corresponding to each treatment and extracted with 1 mL of ethanol 90% for one hour, then centrifuged at 13000g for 5 minutes, the supernatant was discarded and replaced with 1 mL of fresh ethanol 90%, mixed vigorously by vortexing and incubated for another hour, the procedure was repeated for three times until chlorophyll was absent and ethanol supernatant was clear.

Once a clear pellet could be seen, 1 mL of NaOH 1M was added and kept 20 minutes at 80 ^{o}C in a dry bath. After cooling at room temperature the samples were centrifuged at 13000g for 5 minutes and the supernatant transferred to a new 1,5 mL conical tube.

The quantitative determination of the extracted callose was performed in accordance with Kohler A. et al. with some modifications, 33 µL of the supernatant were put in a fresh 1,5 mL conical tube with 467 µL Loading Mixture consisting of 66,7 µL NaOH 1M, 133,3 µL Aniline Blue, 196,7 µL 1M Glycine/NaOH (pH 9,5) and 70 µL HCl 1M. Mixed vigorously by vortexing and incubated for 20 minutes in dry bath at 50 ^{o}C, then cooled to room temperature. (Kohler, Schwindling, & Conrath, 2000)

Total sample fluorescence was read in a Qubit benchtop fluorometer in the protein mode, the results were compared in order to stablish the fold change between treated plants versus non-treated ones.

CHAPTER 5

RESULTS

CHAPTER 5

5. RESULTS

5.1 qPCR RESULTS FOR THE TREATMENTS

After treatment application by spraying to plants, and once 24 or 48 hours have passed, leaves were harvested and freezed with liquid nitrogen, grounded with a mortar and pestle, then the powders were conserved in tubes at -20 ^{o}C.

Each qPCR assay was performed in duplicate, the leaves were collected from two different sets of plants, indicated as sample 1 and sample 2. Special care was taken to prevent results alteration by previous cuts that could trigger plant defense.

Relative quantification system was used to measure genes expression, once the quantitative PCR assay was done, results were tabulated using Genorm normalization method. (Vandesompele & al., 2002)

All the results are expressed as Fold Change, which describes how mRNA levels change from its initial to a new value considering control and treatment substances applied.

5.1.1 FIRST GROUP OF TREATMENTS

For treatment 1, corresponding to *Equisetum arvense* leaves extract, known as “horsetail”; activation of PR1a and PR1b genes was observed after 24 hours; while for SlWRKY genes no activation was appreciated. Figure 41.

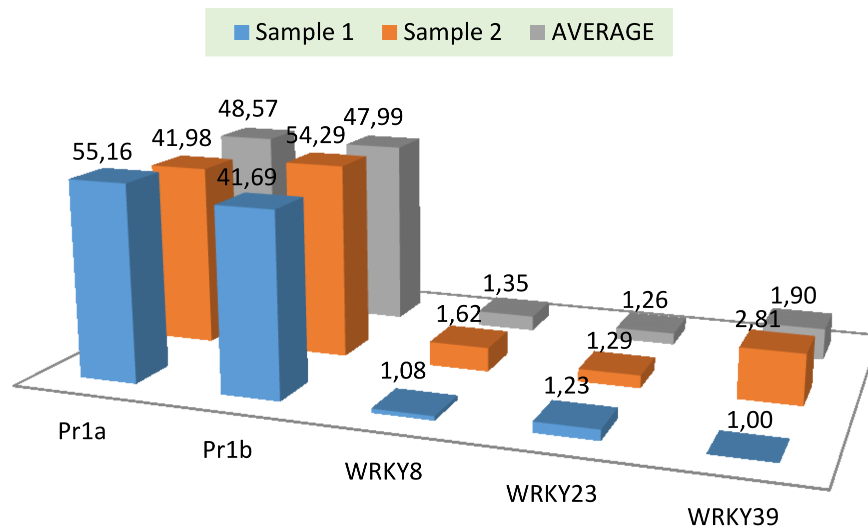


Figure 41. Expression analysis of PR1a, PR1b, SlWRKY8, SlWRKY23 and SlWRKY39 genes, 24 hours after the application of a solution containing an extract of the plant Equisetum arvense.

After 48 hours, activation of PR1b gene decreases from an average of 47,99 to 11,89; while PR1a gene is even higher compared to 24 hours sample. SlWRKY 8 and SlWRKY 23 genes have a small increment from an average of 1,35 and 1,26 at 24 hours to 2,62 and 3,21. Figure 42.

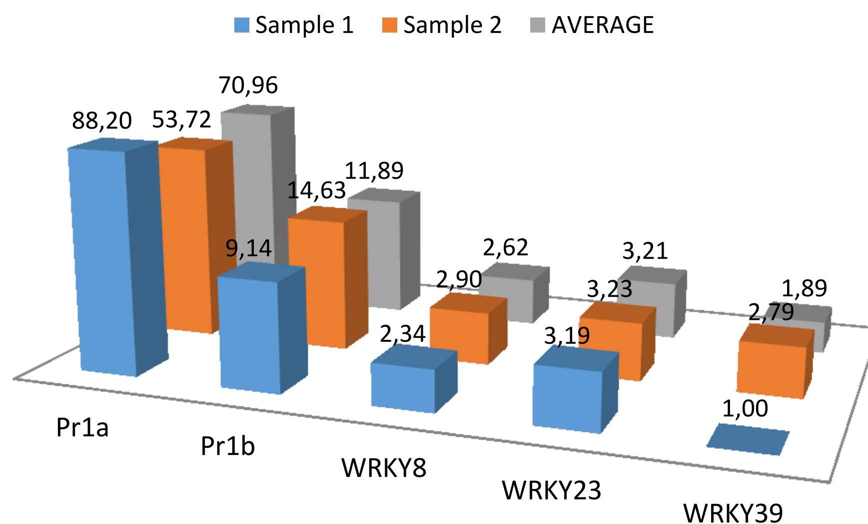


Figure 42. Expression analysis of PR1a, PR1b, SlWRKY8, SlWRKY23 and SlWRKY39 genes, 48 hours after the application of a solution containing an extract of the plant Equisetum arvense.

Considering Chlorine treatment (T2) after 24 hours, activation is observed in the levels of PR1a and PR1b genes, especially for PR1a which is almost eight times higher than PR1b. WRKY genes are not activated visibly as happened with treatment 1. Among them, the average for SlWRKY8 is slightly higher than SlWRKY 23 and SlWRKY 39. Figure 43.

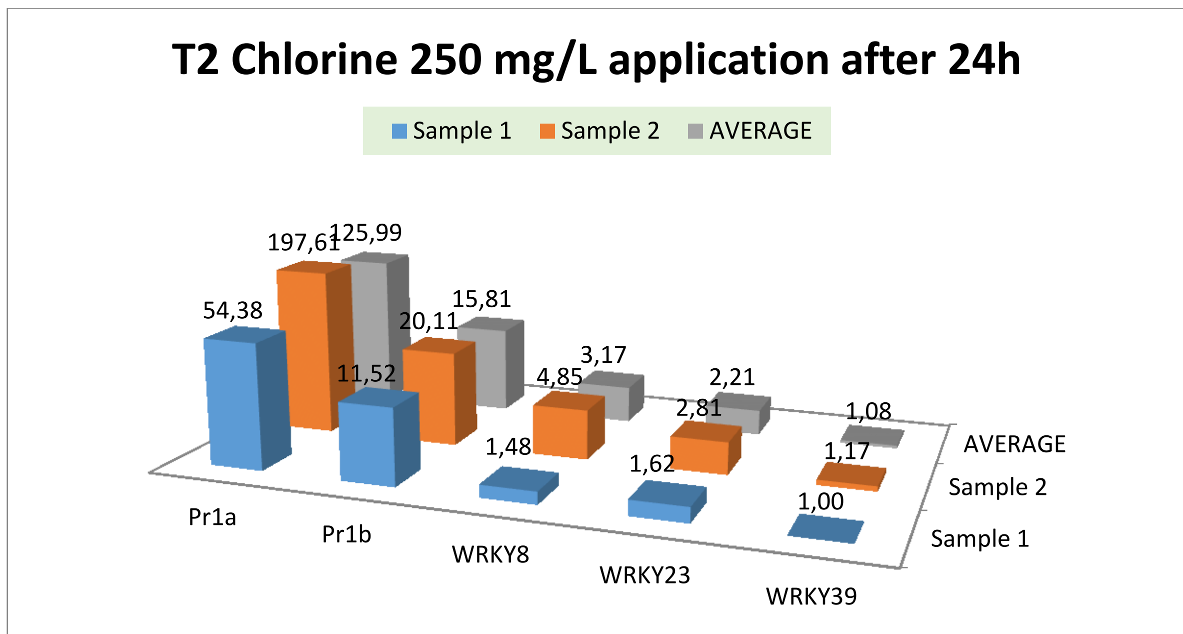


Figure 43. Expression analysis of PR1a, PR1b, SlWRKY8, SlWRKY23 and SlWRKY39 genes, 24 hours after the application of a solution containing 250 mg/L of chlorine.

After 48 hours a large increase is observed for PR1a gene activation; PR1b gene lowers its expression level by one third; whereas, the remaining WRKY genes remain with similar low activation levels. Figure 44.

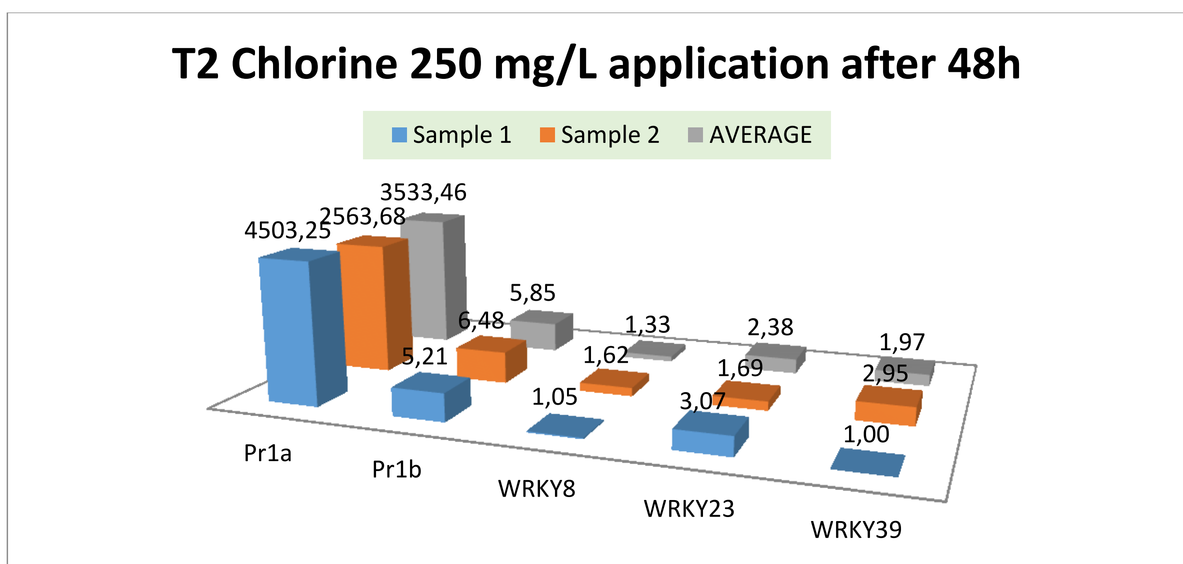


Figure 44. Expression analysis of PR1a, PR1b, SlWRKY8, SlWRKY23 and SlWRKY39 genes, 48 hours after the application of a solution containing 250 mg/L of chlorine.

For treatment 3, commercial Myclobutanyl fungicide, after 24 hours there is no significant activation for all genes, Figure 45. But after 48 hours PR1b and PR1a genes are activated, especially PR1a experiences an increase of more than one hundred percent. While WRKY genes remain almost the same. Figure 46.

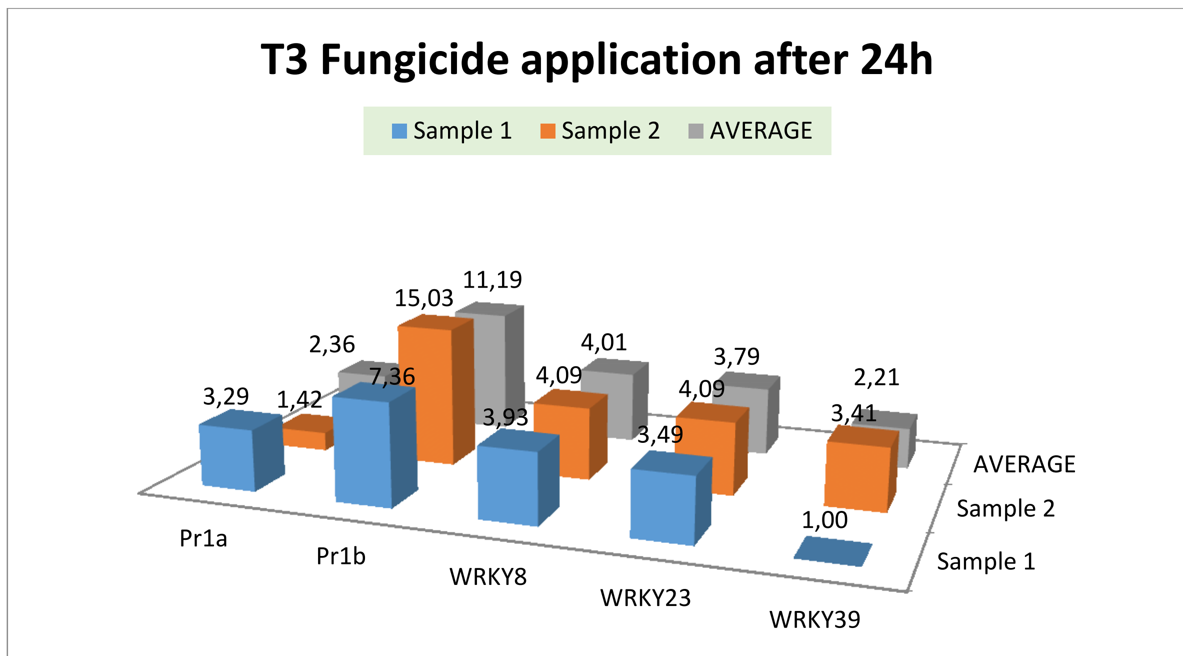


Figure 45. Expression analysis of PR1a, PR1b, SlWRKY8, SlWRKY23 and SlWRKY39 genes, 24 hours after the application of a solution containing myclobutanyl fungicide.

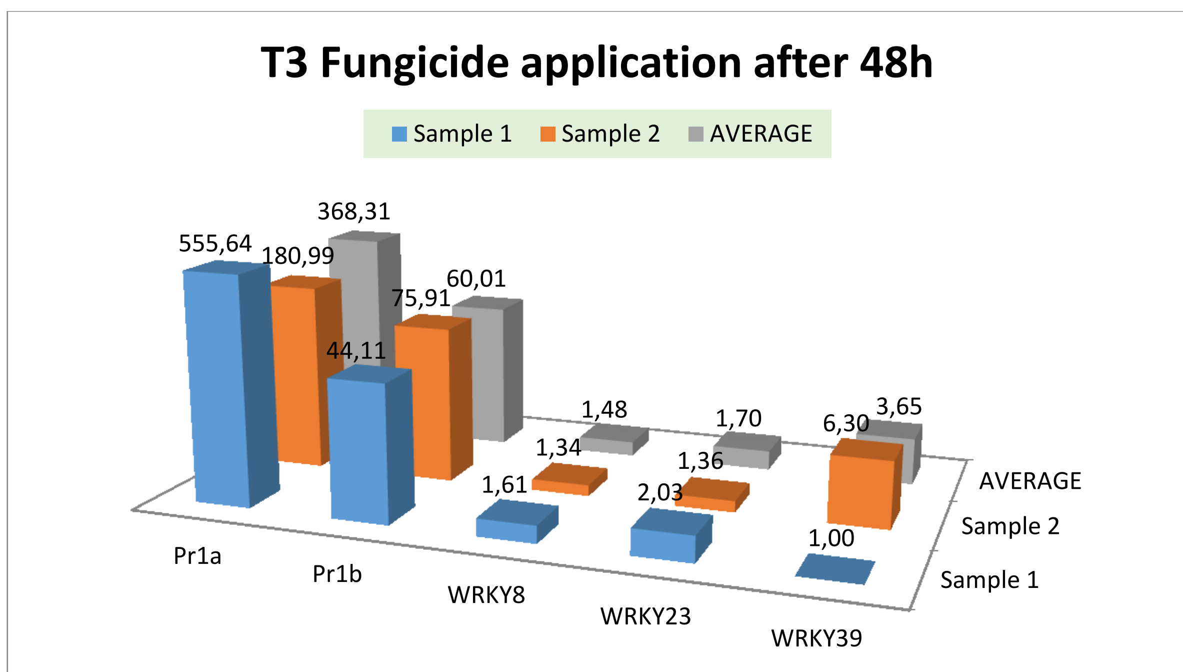


Figure 46. Expression analysis of PR1a, PR1b, SlWRKY8, SlWRKY23 and SlWRKY39 genes, 48 hours after the application of a solution containing myclobutanyl fungicide.

After analyzing these results, the following questions arise:

Are all three applied treatments activating, equally or not, the analyzed genes? Which of the treatments is generating greater activation?

In order to determine if there is a significant difference between the three treatments, a completely random experimental design was applied considering one factor and three levels with a replica.

5.1.1.1 STATISTICAL ANALYSIS OF RESULTS FROM TREATMENTS AT 24 HOURS

Statistical analysis for PR1a gene activation showed a p = 0.252 value, increased relative to alpha = 0.5 value, therefore it is established that there is no significant difference among treatments; and they have a similar statistical behavior.

With regard to PR1b gene activation, a p = 0.024 value is obtained, compared with alfa = 0.05 value, and there is a highly significant difference between the three treatments. By Tukey's test it was determined that treatment with *Equisetum arvense* (T1) is greater than treatment with Chlorine (T2) and this is similar to treatment with fungicide (T3); that is, PR1b gene is more activated with *Equisetum arvense* (T1), as shown in Figure 47.

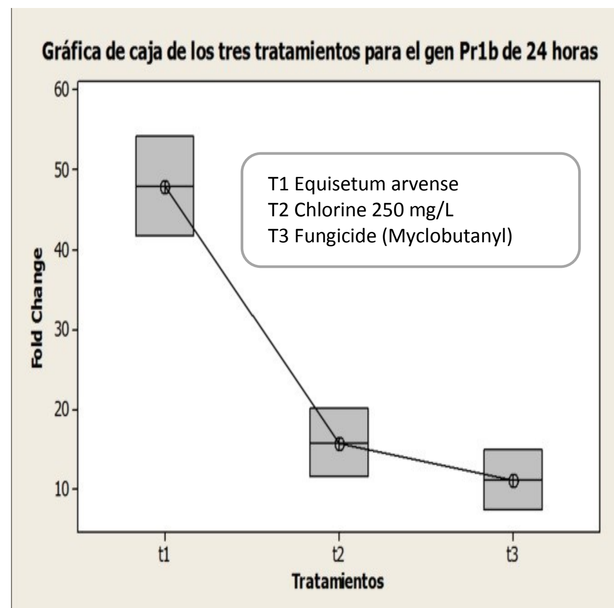


Figure 47. Box chart showing treatments with Equisetum arvense, Chlorine and Fungicide Myclobutanyl after 24 hours, for PR1b Gene.

For SlWRKY 8 gene, according to statistical design, a p = 0.293 value is obtained which is greater than alpha = 0.05 value, therefore there is not significant difference. All three treatments presented similar statistical behavior.

A similar analysis for the SlWRKY23 gene was performed, p = 0.042 value is lower than alpha = 0.05 value, and therefore results are highly significant; that is, the three treatments have a statistically different behavior.

For SlWRKY 39 gene, p = 0.679 that is greater than alpha = 0.05 value, which is not highly significant; that is, all three treatments have a similar statistical behavior.

5.1.1.1 STATISTICAL ANALYSIS OF RESULTS FROM TREATMENTS AT 48 HOURS

The results for PR1a gene activation were statistically analyzed comparing the three treatments T1, T2 and T3, having p = 0.04 value compared with alpha = 0.05; it can be concluded that all three treatments cause a different activation. To determine which has the greatest activation, Tukey's test was used with 95%, showing that T2 with T3 are completely different, with a greater activation for T2; T1 and T3 on the other hand are similar. For these reasons, it can be concluded that T2 is the most active on PR1a gene. Figure 48.

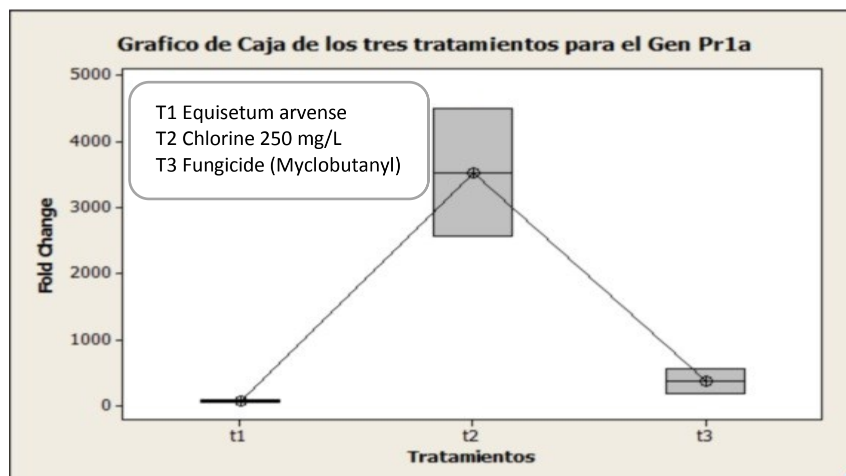


Figure 48. Box chart showing treatments with Equisetum arvense, Chlorine and Fungicide Myclobutanyl after 24 hours, for PR1a Gene.

For PR1b gene, the same statistical analysis was applied, obtaining a p = 0.5 value, compared with an alpha = 0.5 value; establishing that there is a highly significant difference between the three treatments. By Tukey's test it was

determined that T3 has greater effect than T2 and it is similar to T1; meaning that the more active treatment is T3 for PR1b gene, as shown in Figure 49.

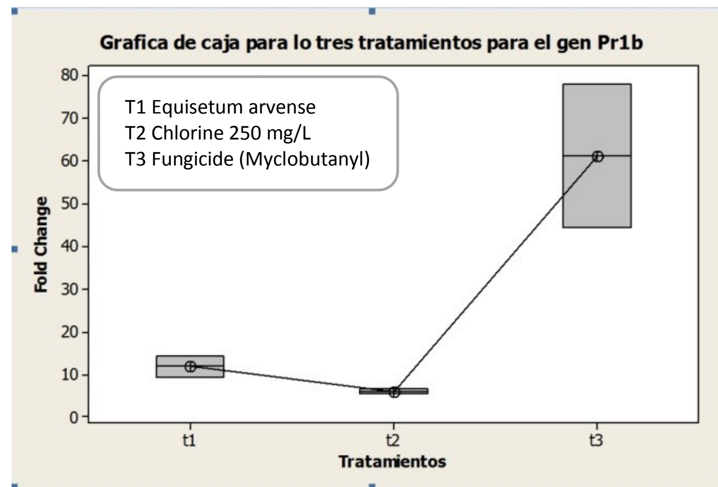


Figure 49. Box chart showing treatments with Equisetum arvense, Chlorine and Fungicide Myclobutanyl after 48 hours, for PR1b Gene.

For SlWRKY 8 gene, according to the statistical design, p = 0.06 value was obtained, which is greater than alpha = 0.05 value; thus the three treatments are not significantly different, statistically the three treatments have a similar behavior.

A p = 0.197 value was obtained for SlWRKY23 gene, which is not highly significant; statistically the three treatments have a similar behavior.

For SlWRKY 39 gene, p = 0.739 value was obtained, which is greater than alpha = 0.05 value; being not significant; statistically the three treatments have a similar behavior.

5.1.2 SECOND GROUP OF TREATMENTS

Treatment corresponding to an aqueous extract of *Capsicum annuum* fruit known as “Rocoto”; activation of PR1b gene was observed after 24 hours; while for Pr1a and SlWRKY genes the activation was not appreciable. Figure 50.

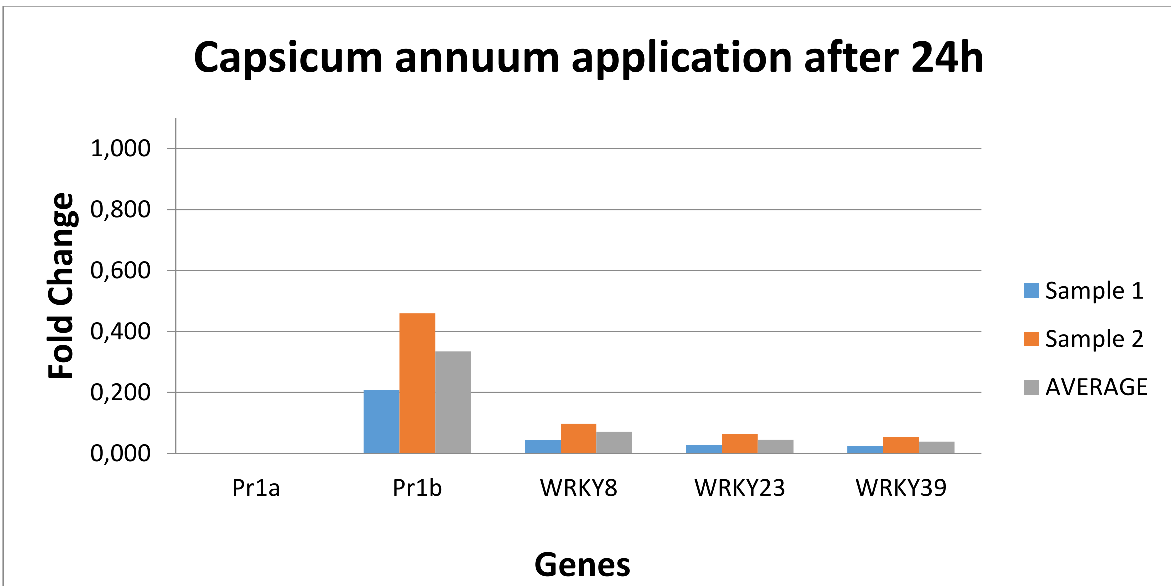


Figure 50. Expression analysis of PR1a, PR1b, SlWRKY8, SlWRKY23 and SlWRKY39 genes, 24 hours after the application of a solution containing chili pepper.

After 48 hours, treatment with aqueous extract of *Capsicum annuum* fruit known as “Rocoto”; had increased the activation of Pr1b gene, while the others remain at very low levels. Figure 51.

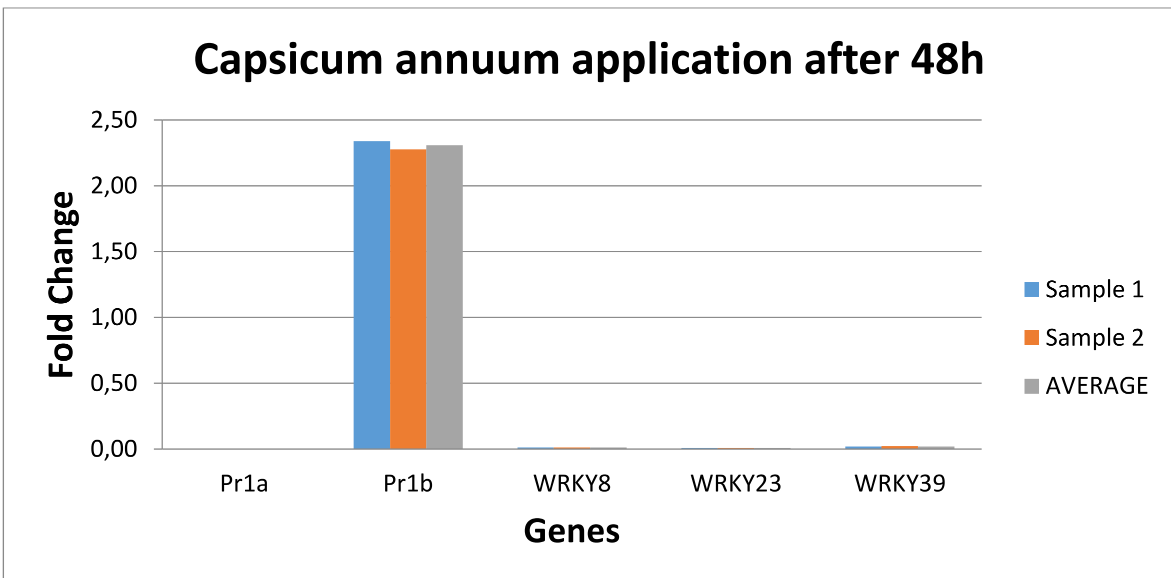


Figure 51. Expression analysis of PR1a, PR1b, SlWRKY8, SlWRKY23 and SlWRKY39 genes, 48 hours after the application of a solution containing chili pepper.

Treatment with Artemisia aqueous extract shows activation of Pr1b gene after 24 hours, while other genes remained at lower levels. Figure 52.

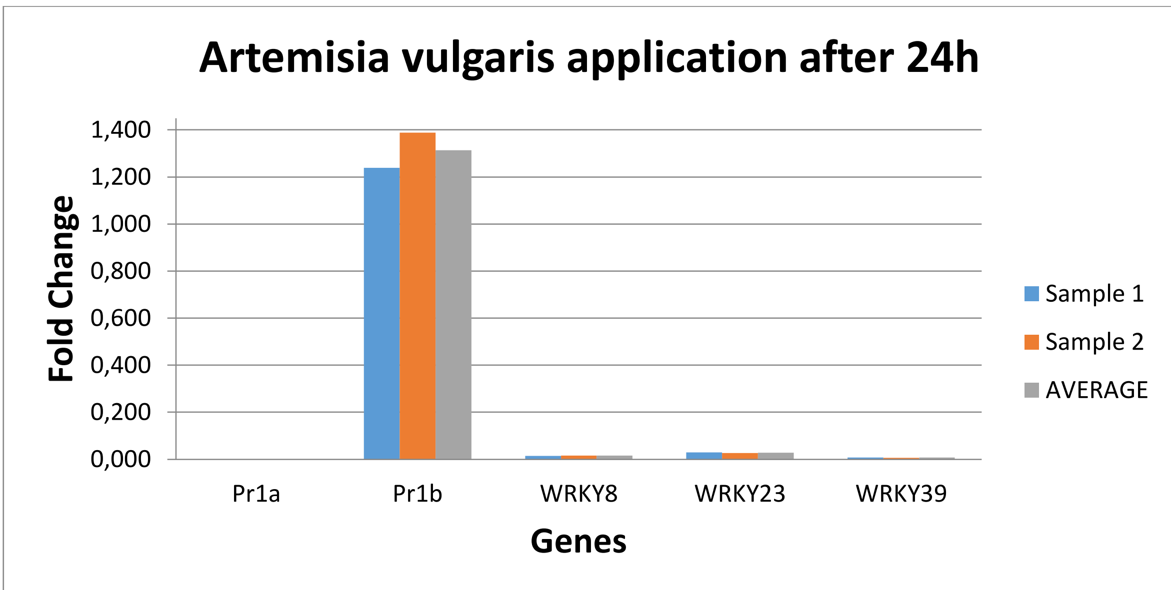


Figure 52. Expression analysis of PR1a, PR1b, SlWRKY8, SlWRKY23 and SlWRKY39 genes, 24 hours after the application of a solution containing artemisa.

After 48 hours, activation of Pr1b gene is increased in a significant manner. Figure 53.

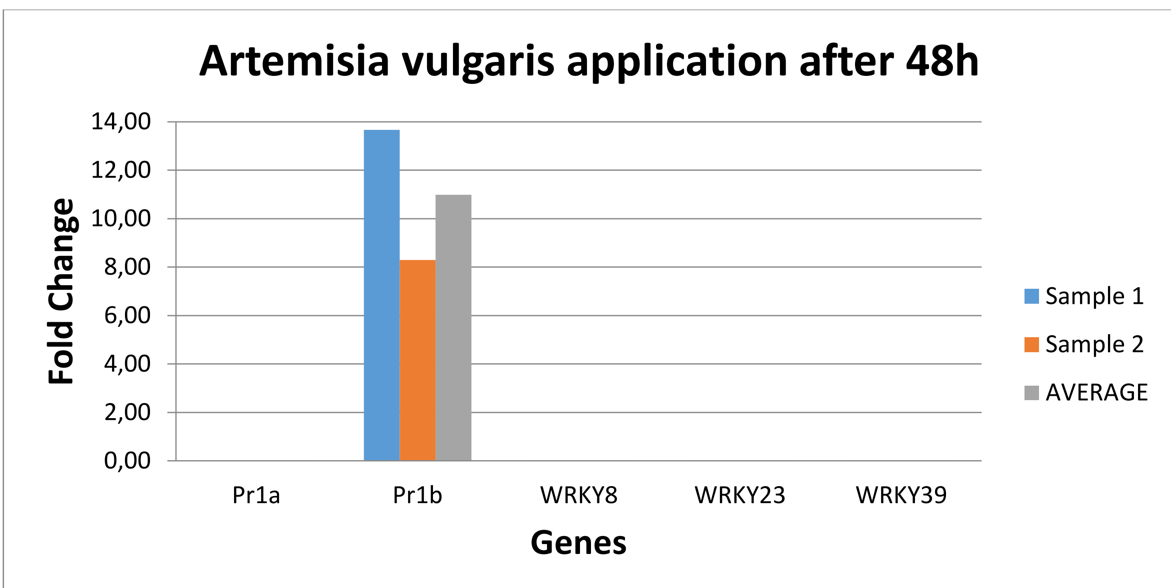


Figure 53. Expression analysis of PR1a, PR1b, SlWRKY8, SlWRKY23 and SlWRKY39 genes, 48 hours after the application of a solution containing artemisa.

The treatment with Chlorine after 24 hours show that Pr1a gene is not activated, and Pr1b, SlWRKY8, SlWRKY23 and SlWRKY39 are slightly activated. Figure 54.

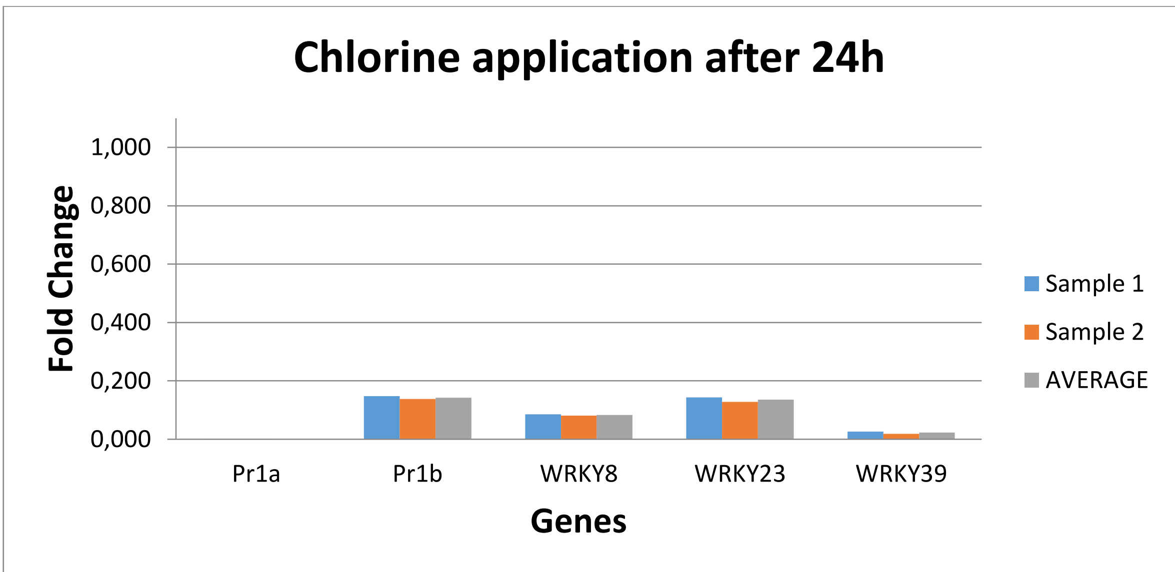


Figure 54. Expression analysis of PR1a, PR1b, SlWRKY8, SlWRKY23 and SlWRKY39 genes, 24 hours after the application of a solution containing chlorine.

48 hours after Chlorine treatment, only Pr1b gene is greatly activated. Figure 55.

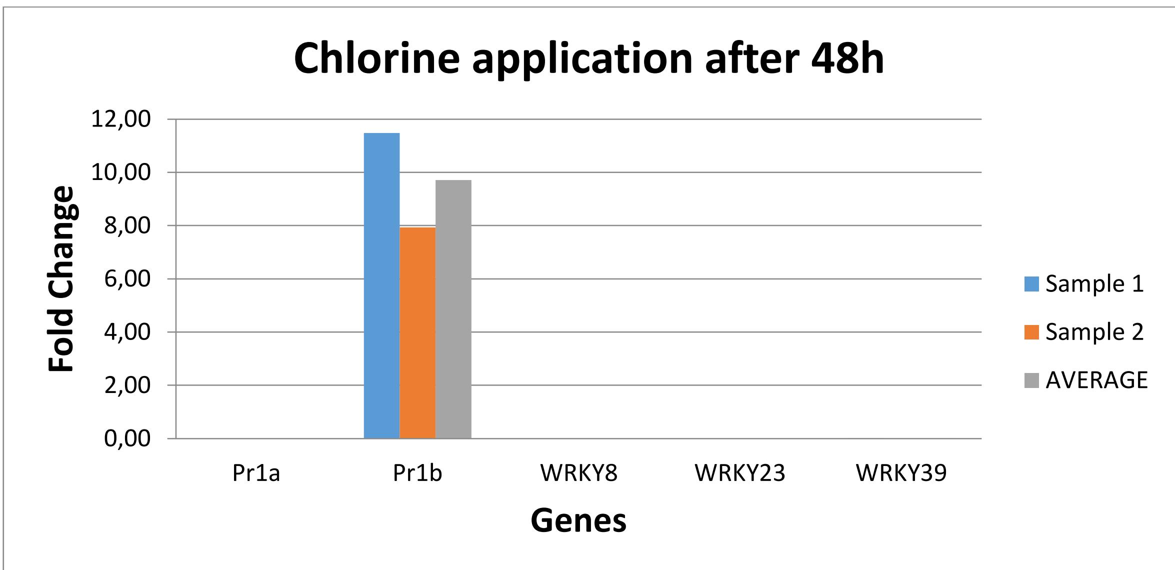


Figure 55. Expression analysis of PR1a, PR1b, SlWRKY8, SlWRKY23 and SlWRKY39 genes, 48 hours after the application of a solution containing chlorine.

Application of deoxicholate aqueous solution activates Pr1b gene. Figure 56.

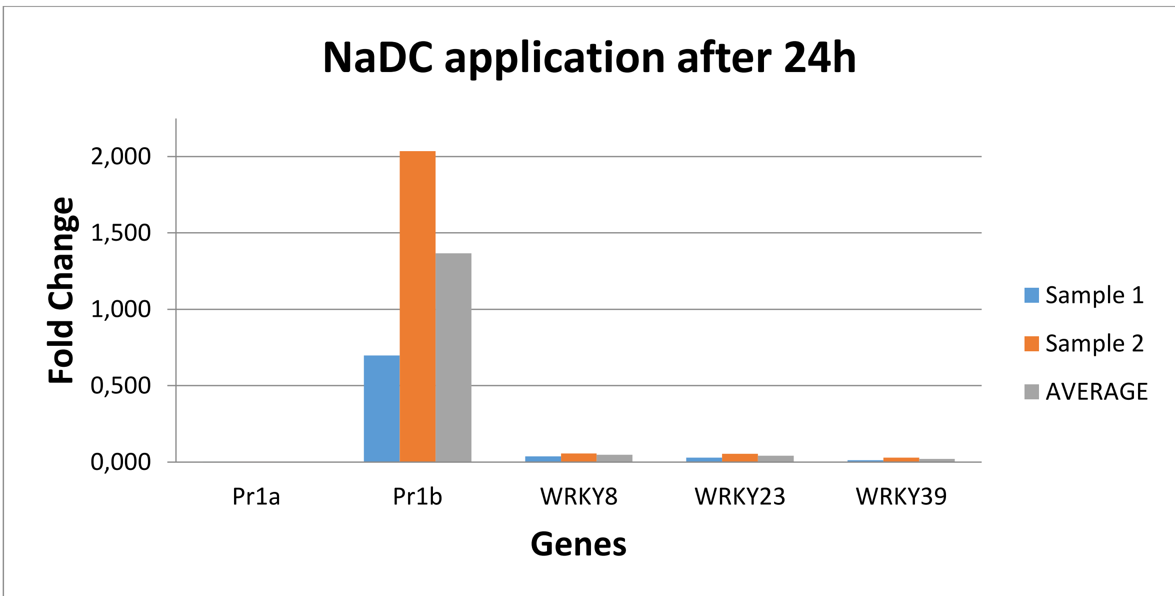


Figure 56. Expression analysis of PR1a, PR1b, SlWRKY8, SlWRKY23 and SlWRKY39 genes, 24 hours after the application of a solution containing sodium desoxicholate.

48 hours after deoxicholate application, gene Pr1b remains at levels similar of those obtained at 24 hours, the other genes tested have lower levels. Figure 57.

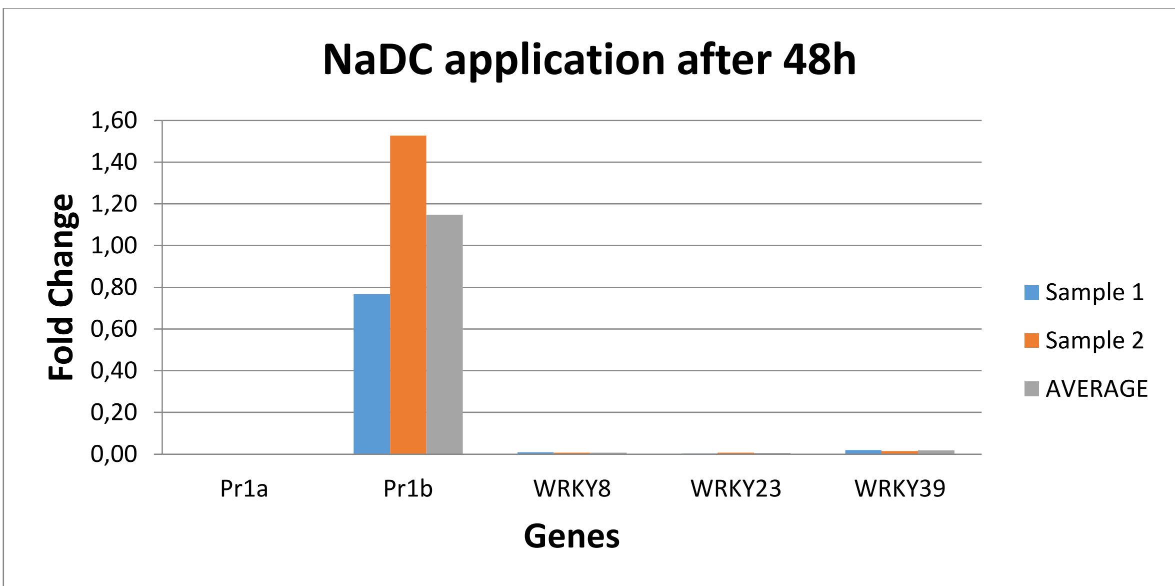


Figure 57. Expression analysis of PR1a, PR1b, SlWRKY8, SlWRKY23 and SlWRKY39 genes, 48 hours after the application of a solution containing sodium desoxicholate.

Treatment with aqueous garlic extract did not produce any important activation of all the tested genes after 24 hours. Figure 58.

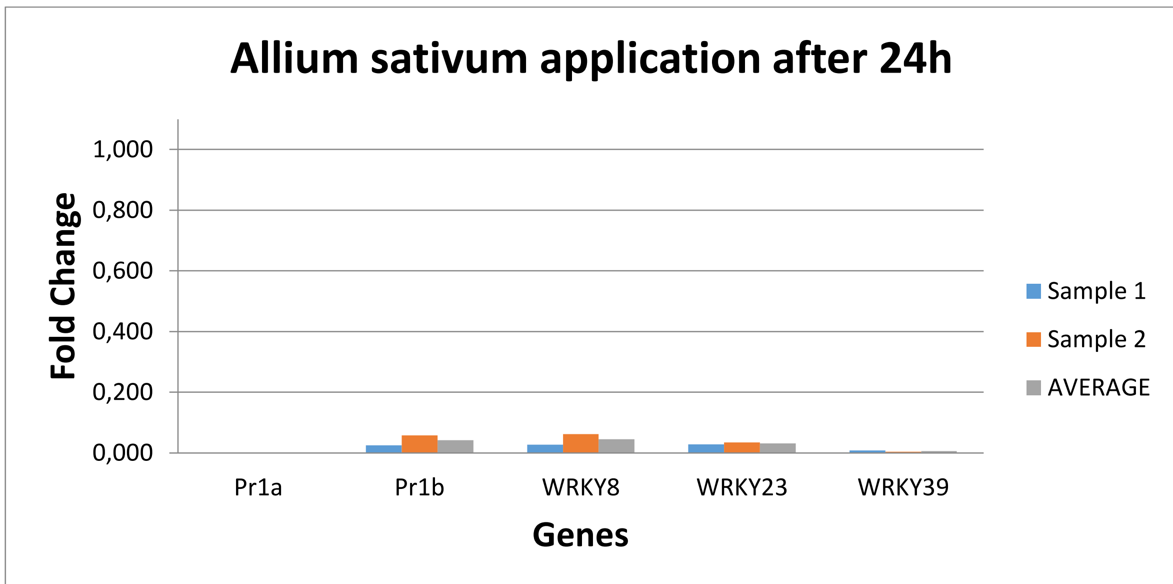


Figure 58. Expression analysis of PR1a, PR1b, SlWRKY8, SlWRKY23 and SlWRKY39 genes, 24 hours after the application of a solution containing garlic.

After 48 hours a slight activation of gene SlWRKY23 is observed while the rest of genes do not show any activation. Figure 59.

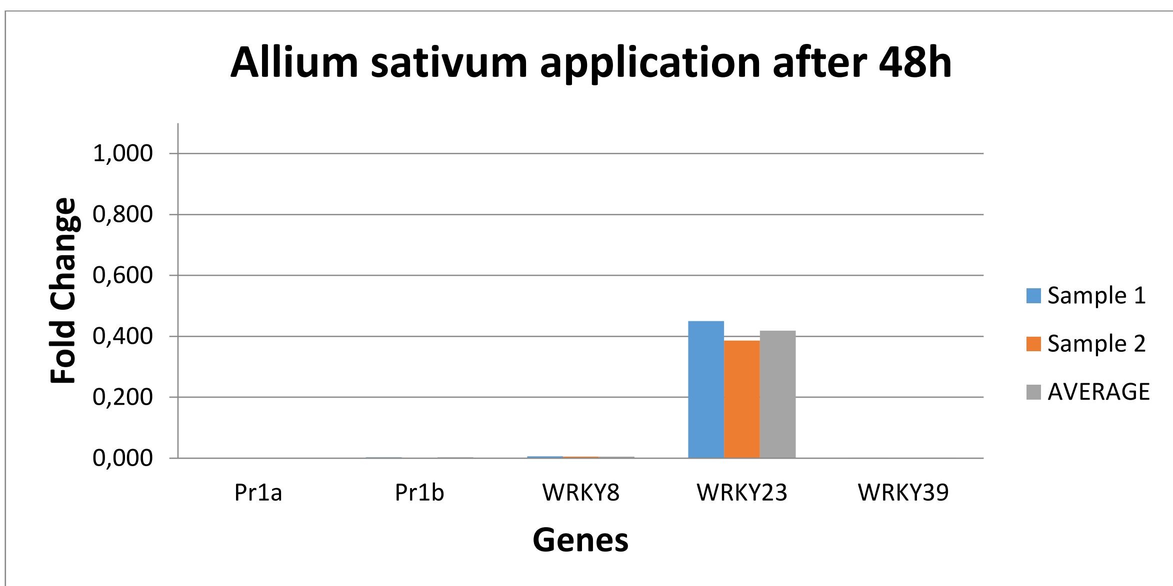


Figure 59. Expression analysis of PR1a, PR1b, SlWRKY8, SlWRKY23 and SlWRKY39 genes, 48 hours after the application of a solution containing garlic.

5.1.2.1 STATISTICAL ANALYSIS OF RESULTS FROM TREATMENTS AT 24 AND 48 HOURS

Analysis made of data obtained both for 24 and 48 hours comply the assumptions of normality, constant variance and independence, thus a completely random design with one input factor, five levels and one output value was applied.

It can be seen in boxplot Figure 60, that all are overlapping, which means they have a similar behavior; this information is ratified through variance analysis ANOVA with p = 0.586 value higher than alpha = 0.05 value.

The differences between these treatments are not highly significant meaning that they act in a similar way.

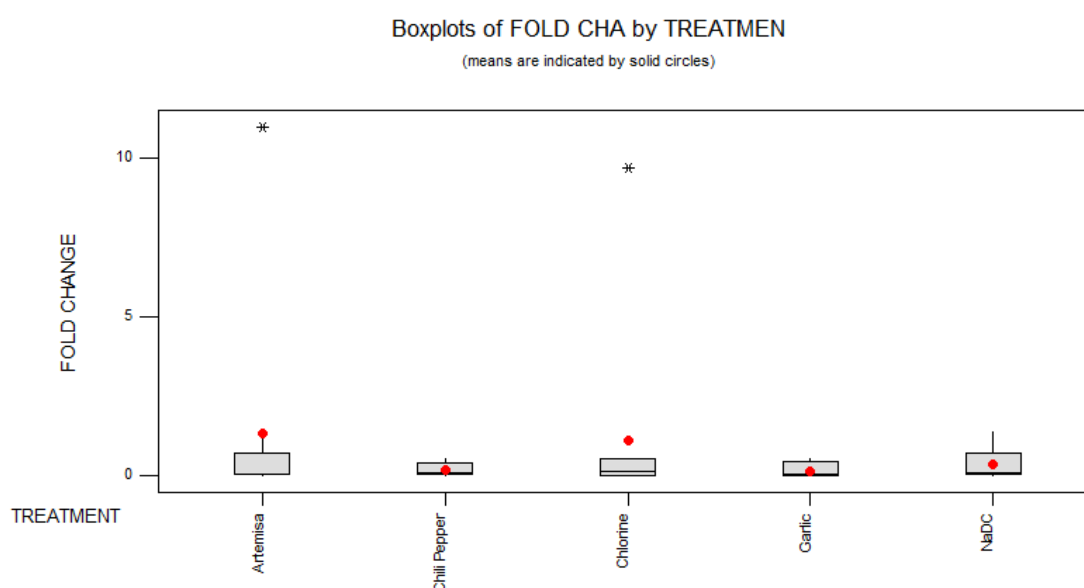


Figure 60. Boxplots of Fold Change by Treatment applied at 24 hours.

Analysis of results obtained at 48 hours indicate they have a similar behavior as the ones from 24 hours, in the box chart an overlap between all treatments boxes is observed. Besides there is a greater variation in *Artemisia* and chlorine treatments. Figure 61.

To confirm the box charts obtained results, a completely random design was made, similar to the one performed for 24 hours, in addition the obtained data comply with the model assumptions, resulting in p = 0.622 value greater than alpha = 0.05 value, stating that results are not highly significant, thus acting in the same way.

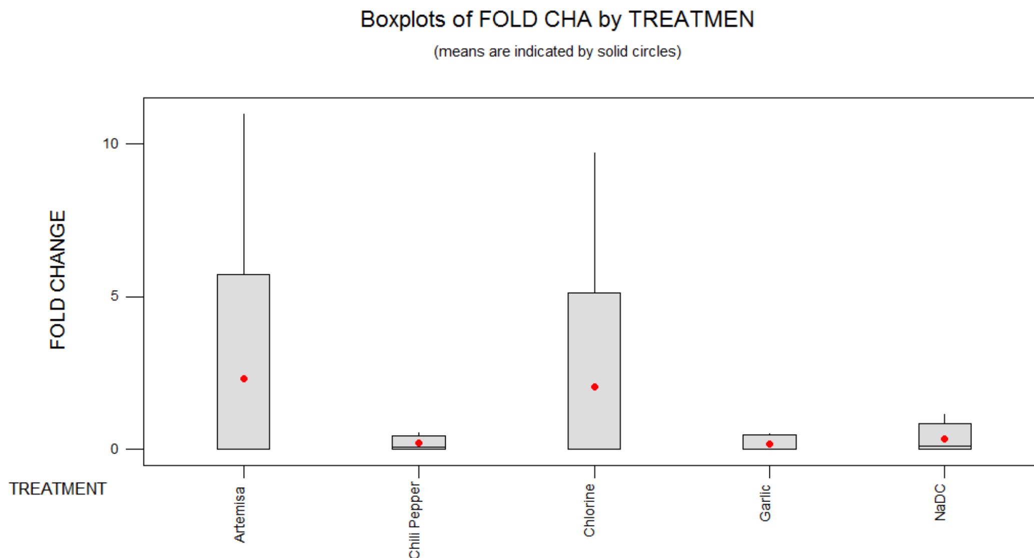


Figure 61. Boxplots of Fold Change by Treatment applied at 48 hours.

5.1.3 THIRD GROUP OF TREATMENTS

The results obtained with rue (*Ruta graveolens*), clove (*Syzygium aromaticum*) and chili pepper (*Capsicum annuum*) aqueous extracts evidence the best activation values of endogenous defenses in tomato plants, similar results were obtained with tobacco model plants. This levels are comparable to positive control solutions (Sodium Deoxycholate and diluted Chlorine).

For some of this natural extracts the overexpression or enhancement can also be observed with the second treatment after 15 days, a phenomenon also observed with the positive control. (Zarattini & al., The use of ECAS in plant protection: a green and efficient antimicrobial approach that primes selected defense genes, 2015)

It is important to highlight that negative control treatment (water) did not activate the genes object of the present study in any case.

5.1.3.1 STATISTICAL ANALYSIS OF RESULTS FROM TREATMENTS AT 24 AND 48 HOURS

The expression of PR1 gene exhibits an increase after 24 hours upon treatments. Expression levels are higher for rue (*Ruta graveolens*), clove (*Syzygium

aromaticum) and chili pepper (*Capsicum annuum*) compared to positive control Sodium Deoxycholate (NaDC), and to a lesser extent than positive control diluted Chlorine (HClO). Figure 62.

When the expression of PR1 gene is analyzed at 48 hours, an increase can be observed for treatments with chili pepper (*Capsicum annuum*) and clove (*Syzygium aromaticum*), while the expression level with rue (*Ruta graveolens*) drops slightly. In any case, they are superior to positive control Sodium Deoxycholate (NaDC) and inferior to positive control diluted Chlorine (HClO). Figure 62.

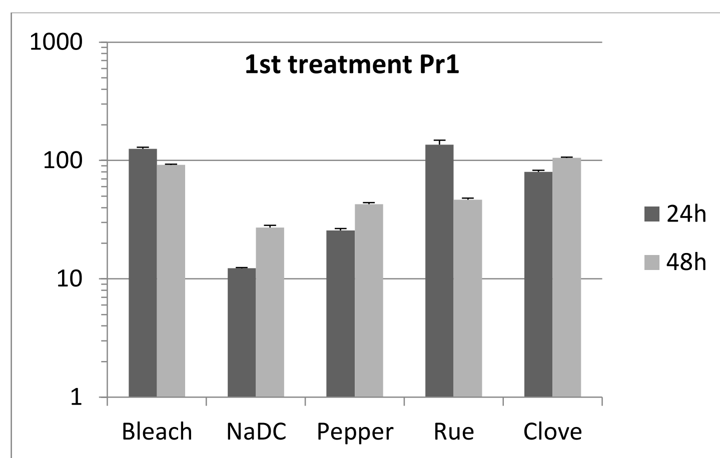


Figure 62. Levels of gene induction (fold change) for PR1b gene 24 and 48 hours after the treatment of tomato plants with different extracts, expressed in relation to the level of gene expression in negative control plants (treated with water).

PR2 (glucanase) gene shows an activation level lower than PR1. Comparing treatments applied to plants at 24 hours, chili pepper (*Capsicum annuum*) and rue (*Ruta graveolens*) PR2 gene activation resulted similar to positive control Sodium Deoxycholate (NaDC) and inferior to positive control diluted chlorine (HClO); while clove (*Syzygium aromaticum*) treatment activated PR2 gene more effectively than positive control diluted chlorine (HClO). Figure 63.

ß-1,3-glucanases are well known pathogenesis related proteins and their expression is induced by different kind of pathogens and defense-related phytohormones (Spoel & Dong, 2012), furthermore transgenic plants studies have shown that overexpressing PR2 glucanases increased disease resistance and delayed symptoms (Balasubramanian & al., 2012) Natural extracts used in this investigation are being able to stimulate glucanases accumulation and to increase

plant defenses before an infection, therefore could protect tomato plants against biotic stress.

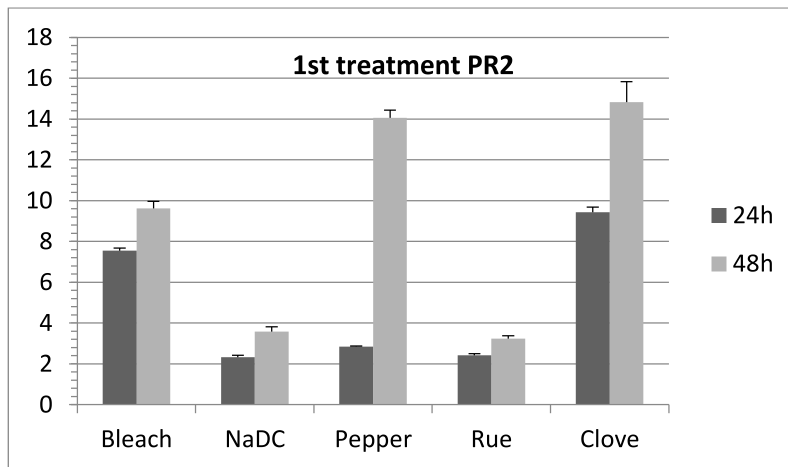


Figure 63. Levels of gene induction (fold change) for glucanase gene of tomato plants treated with different extracts 24 and 48 hours after treatment, expressed in relation to the level of gene expression in negative control plants (treated with water).

Analyzing expression levels for PR3 (chitinase) gene, evidenced that treatments with chili pepper, rue and clove provided similar expression levels after 24 hours, lower than positive controls diluted chlorine (HClO) and Sodium Deoxycholate (NaDC). Figure 64.

Considering results after 48 hours, an increase in expression levels was observed for chili pepper, rue and clove treatments, being higher than Sodium Deoxycholate (NaDC) positive control but lower than diluted chlorine (HClO) positive control.

The simultaneous presence of both types of pathogen defense proteins has been already associated with increased resistance to diseases (Balasubramanian & al., 2012) and it is a common feature in primed plants and systemic acquired resistance. (Conrath, 2009)

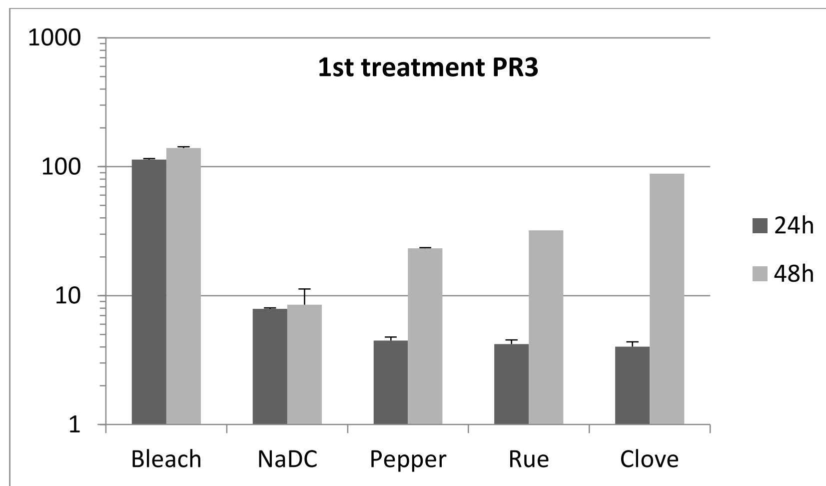


Figure 64. Levels of gene induction (fold change) for tomato chitinase gene 24 and 48 hours after treatment with different extracts, expressed in relation to the level of gene expression in negative control plants (treated with water).

To further study the role of plant extracts activating plant defenses, the expression profiles of two plant resistance regulators belonging to mitogen-activated protein kinase MAPK group were considered, tomato MAPK3 and MAPK6 were chosen by virtue of their known role in hypersensitive and pathogen resistance regulation. (Stulemeijer & al., 2007) (Kong & al., 2012)

Expression levels achieved 24 hours after treatments were inferior to diluted chlorine (HClO) and Sodium Deoxycholate (NaDC) positive controls; after 48 hours these levels of expression became very similar to positive controls, especially chili pepper (*Capsicum annuum*) treatment, while rue (*Ruta graveolens*) and clove (*Syzygium aromaticum*) had lower levels of gene expression. Figure 65.

Therefore regulatory proteins belonging to MAPK group appeared rapidly and transiently up-regulated upon treatment.

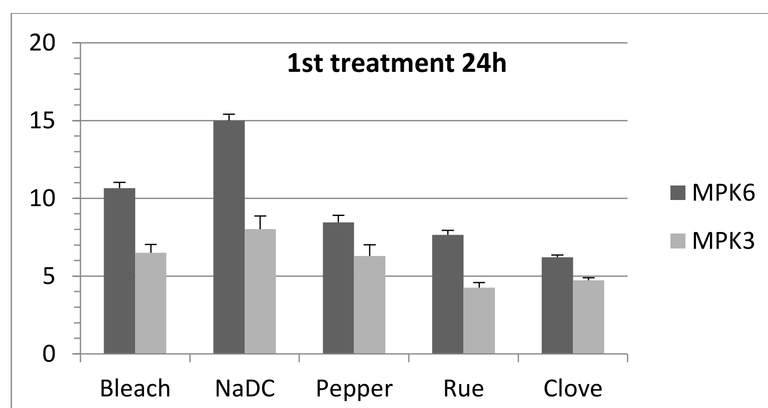


Figure 65. Levels of gene induction (fold change) of tomato MPK6 MPK3 genes 24 and 48 hours after treatment with different extracts, expressed in relation to the level of gene expression in negative control plants (treated with water).

5.1.3.2 STATISTICAL ANALYSIS OF RESULTS FROM TREATMENTS AT 24 AND 48 HOURS, APPLIED 15 DAYS AFTER THE FIRST SPRAYING

A new treatment with the same substances was applied to plants of the first experiment, 15 days after the first treatment.

In 24 hours samples PR1 gene expression levels for chili pepper (*Capsicum annuum*), rue (*Ruta graveolens*) and clove (*Syzygium aromaticum*) treatments were superior than Sodium Deoxycholate (NaDC) positive control but lower than diluted Chlorine (HClO) positive control. In 48 hours samples, the levels of expression were almost halved for chili pepper treatment (*Capsicum annuum*), and more than 15 times higher for rue (*Ruta graveolens*), whereas with clove (*Syzygium aromaticum*) the levels suffered a slight decrease. Figure 66.

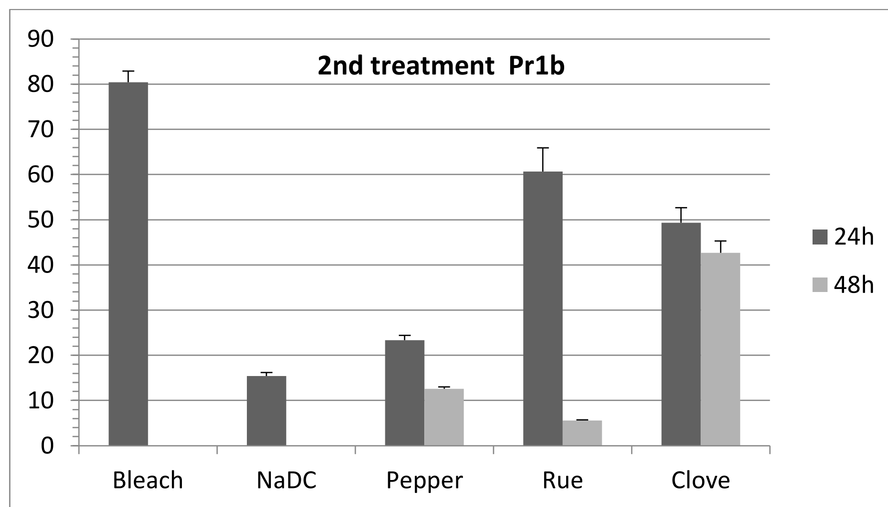


Figure 66. Levels of gene induction (fold change) for PR1b gene in tomato plants after 24 and 48 hours of a second treatment with different extracts 15 days after the first, expressed in relation to the level of gene expression in negative control plants (treated with water).

The expression levels of PR2 gene 24 hours after treatment with chili pepper and clove plant extracts were higher than positive controls. Whereas gene expression obtained with rue was 10 times higher than positive control. 48 hours upon treatment gene expression obtained with chili pepper remained similar to that at 24 hours, while it decrease 8 times for rue. Clove treatment induced a substantial increase in gene expression levels. Figure 67.

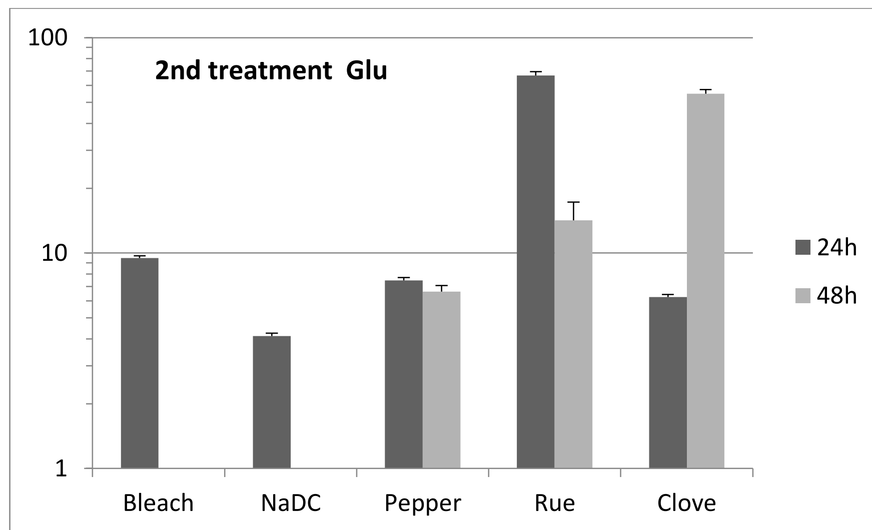


Figure 67. Levels of gene induction (fold change) for Glucanase gene in tomato plants after 24 and 48 hours of a second treatment with different extracts 15 days after the first, expressed in relation to the level of gene expression in negative control plants (treated with water).

Expression levels for PR3 gene 24 hours after the second treatment were lower than positive controls for chili pepper and clove, while rue provided an intermediate expression level between the two positive controls. After 48 hours the expression levels decreased for treatments with chili pepper, clove and considerably for rue. Figure 68.

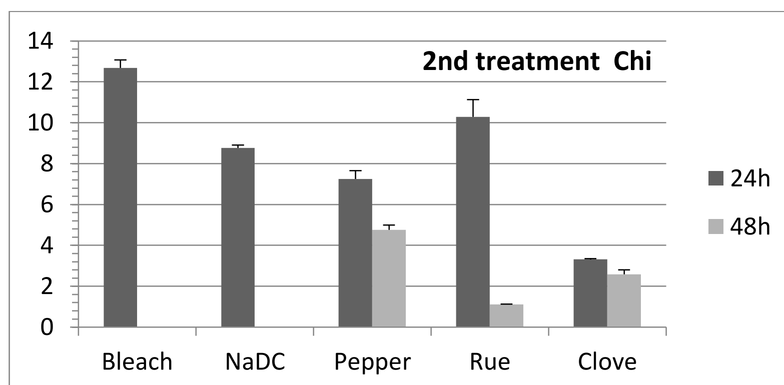


Figure 68. Levels of gene induction (fold change) for chitinase gene in tomato plants 24 and 48 hours after a second treatment with different extracts 15 days after the first, expressed in relation to the level of gene expression in negative control plants (treated with water).

5.2 IN VIVO LEAF INFILTRATION ASSAY

To confirm molecular data obtained, natural extracts that presented better gene activation were used in leaf infiltration experiments both in tomato and tobacco wild type leaves. The extract infiltration was performed in the upper leaf using a

syringe, then leaf phenotype was analyzed one or two days later. The model plant chosen was tomato considering its agronomic importance and economical value.

The necrosis area in leaves infiltrated with extracts is a typical manifestation of hypersensitive response HR in defense against pathogens. Figure 69. This type of response was observed even with control solutions (chlorine and NADC as positive controls), but not with water alone (negative control). These experiments demonstrated that extracts of pepper “Rocoto”, rue and clove, identified by qPCR, were able to activate hypersensitive response with programmed cell death, a sign of high efficiency in immune response functional activation.

These data indicate that this three analyzed extracts represent good candidates that could be used as priming activators in plants, as a pre-activation of endogenous defenses against pathogens.

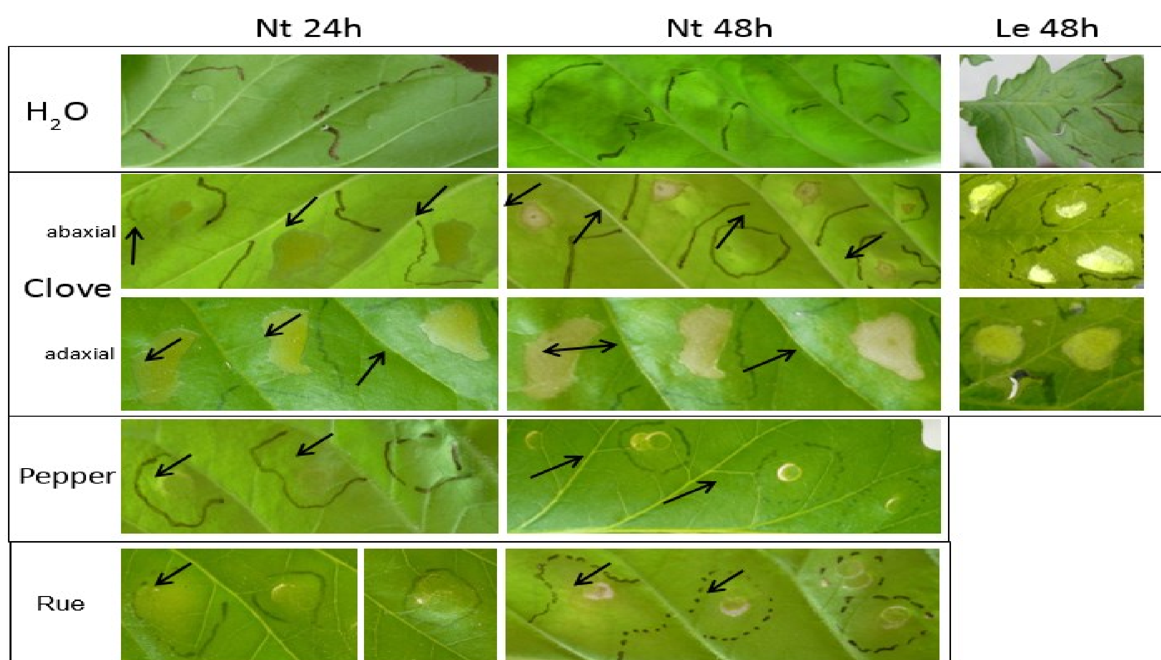


Figure 69. Infiltration experiments in tomato (Le) and tobacco (Nt) plant leaves with natural extracts. The hypersensitive response induction (necrosis areas, arrows) is present only with extracts but not with water.

5.3 HISTOCHEMICAL GUS ASSAY

PR1-GUS tobacco transgenic plants were grown and treated with the same natural extracts used on tomato plants. This model plant (Grüner & U.M., 1994) allows to monitor PR1 promoter activity by means of GUS reporter gene, whose corresponding protein can be easily detected by histochemical staining procedure. Tobacco healthy PR1-GUS plants were sprayed with chili pepper, wild rue and

clove extracts in the same conditions used for tomato. 24 and 48 hours after treatment leaf samples were collected and subjected to X-gluc staining, specific for GUS activity. Upon discoloration, the blue staining was evaluated and photographed.

PR1a-GUS transgenic plants treated with different solutions (BTH, 140 mg/L was used as positive control) were tested for GUS activity by histochemical assay carried out with a modified protocol. (Degrave & al., 2008)

Tobacco leaves were harvested 24 and 48 hours after the treatment and incubated in 50 mM Na_2HPO_4 and 10 mM EDTA, pH 7.0 containing X-gluc substrate (0.05% w/v). Staining was performed in darkness at 37 °C for 16 to 20 hours. To remove chlorophyll, leaves were washed several times with hot 70% ethanol. Figures 70 and 71 evidence Histochemical Gus Assay results considering the first treatment and the second one 15 days after the first on tomato and tobacco plants.

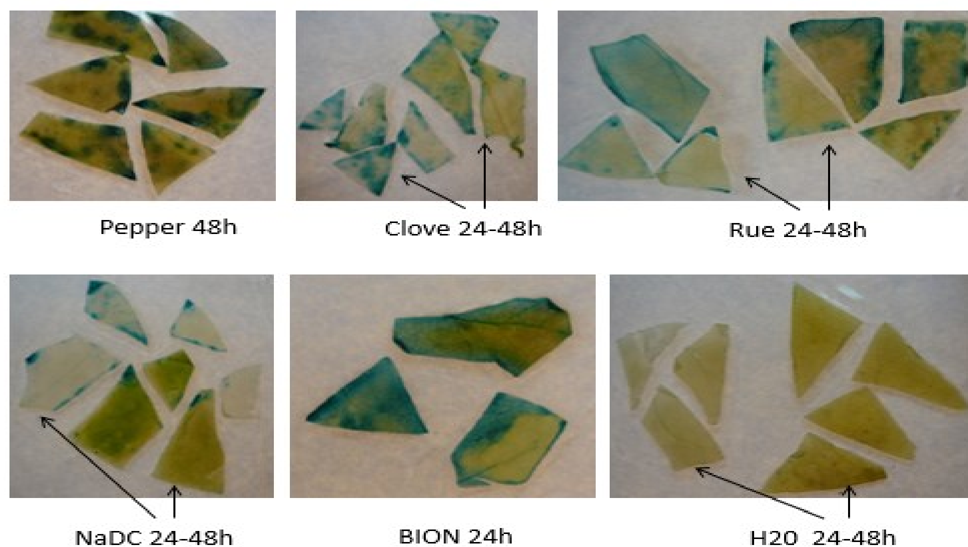


Figure 70. GUS-assays 24h or 48h after each treatment on PR1-GUS tobacco transgenic plants, 1st treatment.

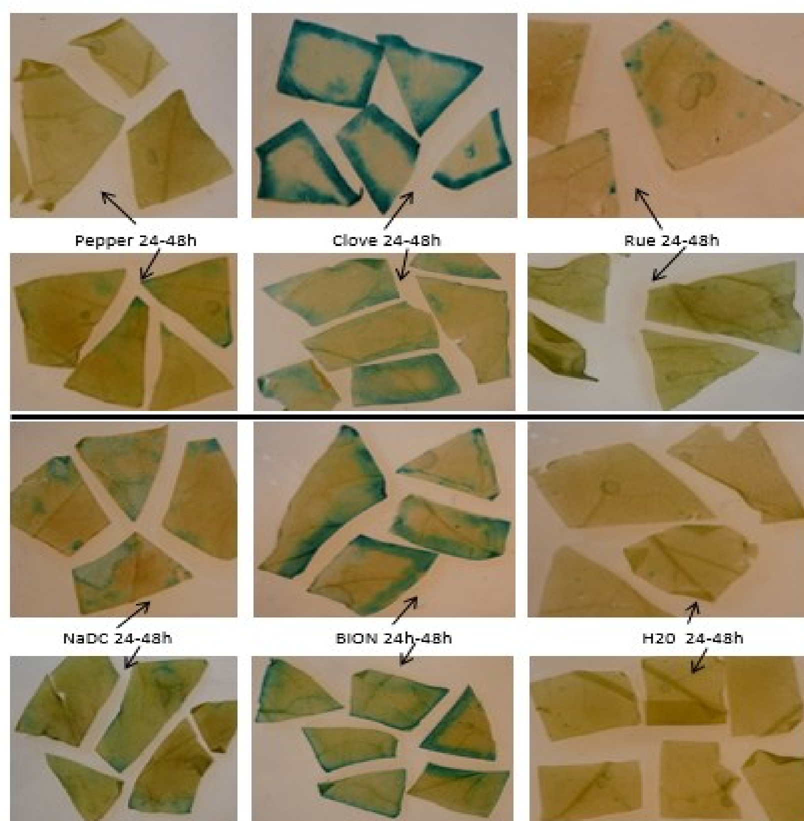


Figure 71. GUS-assays 24h or 48h after each treatment on PR1-GUS tobacco transgenic plants, 2nd treatment 15 days after the first.

5.4 HPLC SALICYLIC ACID ASSAY

The results obtained by HPLC analysis, 24 and 48 hours after treatment with plant extracts are summarized in Figure 72.

Results at 24 hours for treatments with rue and chili pepper produced salicylic acid values four times higher than negative control (without any treatment), whereas clove and water also activated salicylic acid production but in lower proportion. Positive control Chlorine activated higher salicylic acid production, five times the negative control, whereas NaDC maintained values similar to those produced with water treatment.

Salicylic acid production at 48 hours were maintained at similar levels to the initials for water and clove, slightly decreased for treatments with chili pepper, chlorine, rue and NaDC.

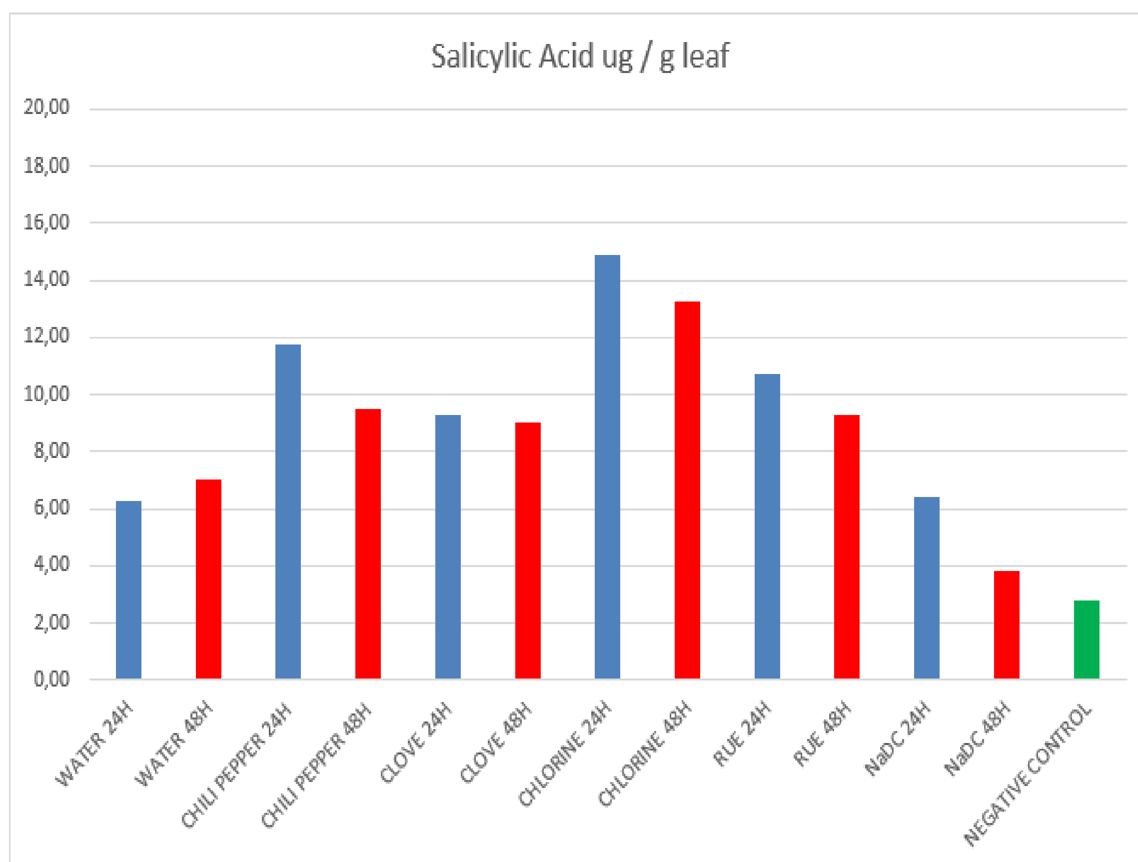


Figure 72. Salicylic Acid results from samples after 24 and 48 hours.

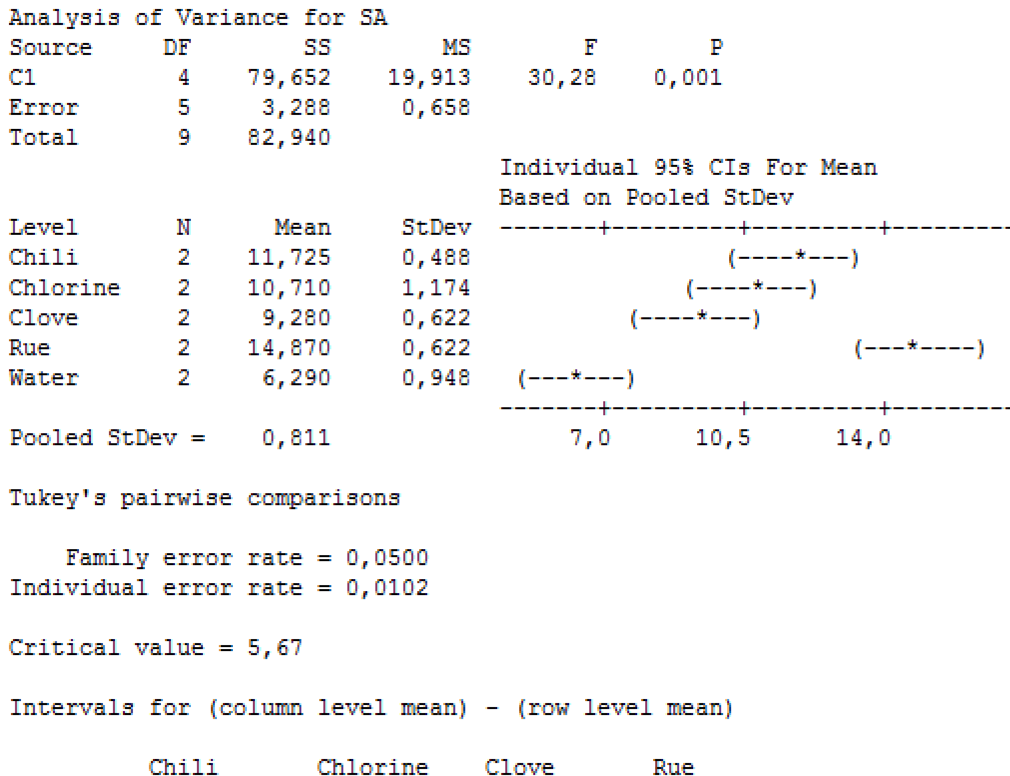
5.4.1 HPLC SALICYLIC ACID ASSAY STATISTICAL ANALYSIS AT 24 HOURS

According to Tukey's test, considering alfa = 0.05 value it can be stated that compared to water, rue produced an increased activation; while chili pepper, chlorine and clove had similar behaviors. Figure 73.

Residuals vs Fits for SA

Residuals vs Order for SA

One-way ANOVA: SA versus C1



```
Analysis of Variance for SA
Source     DF        SS        MS        F        P
C1          4    79,652    19,913    30,28    0,001
Error       5     3,288     0,658
Total       9    82,940
                                   Individual 95% CIs For Mean
                                   Based on Pooled StDev
Level       N      Mean     StDev  -------+---------+---------+---------
Chili       2    11,725     0,488                (----*---)
Chlorine    2    10,710     1,174             (----*---)
Clove       2     9,280     0,622         (----*---)
Rue         2    14,870     0,622                          (---*----)
Water       2     6,290     0,948   (---*---)
                                   -------+---------+---------+---------
Pooled StDev =    0,811                 7,0      10,5      14,0

Tukey's pairwise comparisons

    Family error rate = 0,0500
Individual error rate = 0,0102

Critical value = 5,67

Intervals for (column level mean) - (row level mean)

          Chili       Chlorine     Clove        Rue
```

Figure 73. ANOVA analysis of Salicylic Acid production versus treatments applied: Chili Pepper, Chlorine, Clove, Rue and Water, 24 hours after.

Box plot analysis confirms that rue extract was able to activate highest salicylic acid SA accumulation, the box is located at the top of the chart without overlapping other treatments. Water treatment generated low salicylic acid SA quantities while the other three treatments (Clove, Chili and Chlorine) act similarly having overlaped boxes in the chart. Figure 74.

It is further noted that Chlorine and water treatments had a greater variability.

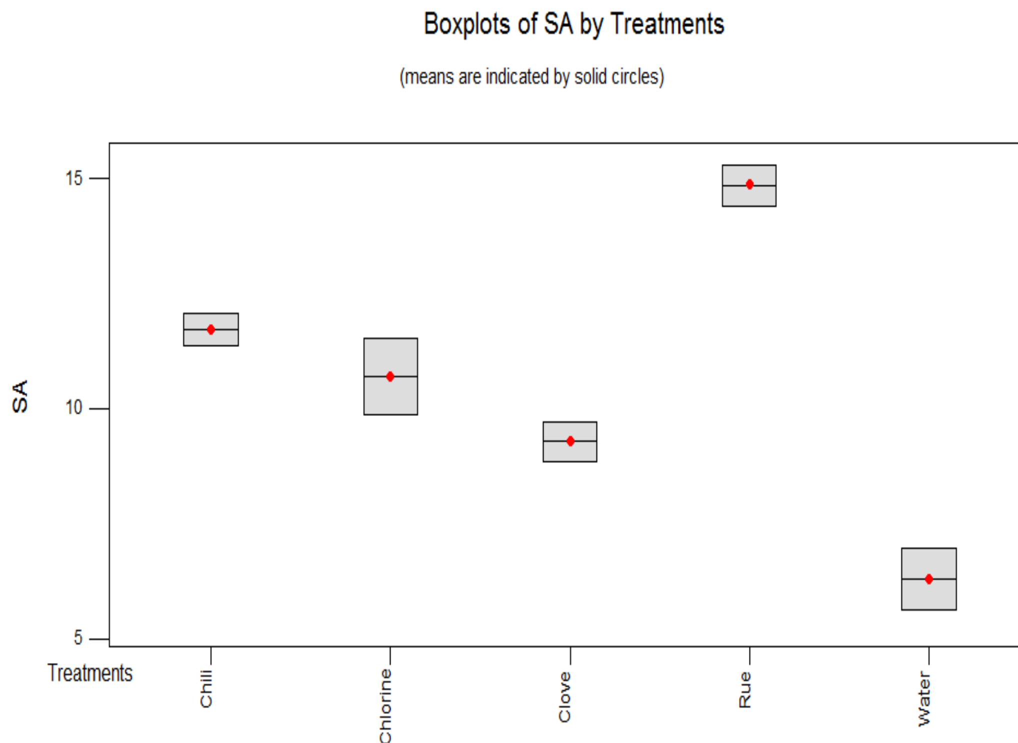


Figure 74. Box Chart of Treatments applied versus Salicylic Acid production, 24 hours after.

5.4.2 HPLC SALICYLIC ACID ASSAY STATISTICAL ANALYSIS AT 48 HOURS

According to normal chart test data, assumptions of normality, constant variance and independence were met. Therefore a completely randomized design was designed which resulted not significant, thus null hypothesis is true stating that treatments do not behaved differently. (p value = 0.676 and alpha = 0.05). Figure 75.

One-way ANOVA: AS versus Treatments

```
Analysis of Variance for AS
Source     DF        SS        MS        F        P
Treatmen    4      40,6      10,1     0,61    0,676
Error       5      83,6      16,7
Total       9     124,2
                                   Individual 95% CIs For Mean
                                   Based on Pooled StDev
Level       N      Mean     StDev  -+---------+---------+---------+-----
CHILI PE    2     9,520     1,994      (------------*-----------)
CHLORINE    2    13,245     8,323             (-----------*-----------)
CLOVE 48    2     9,040     2,418      (-----------*-----------)
RUE 48H     2     9,280     2,121      (-----------*------------)
WATER 48    2     7,035     0,163  (------------*-----------)
                                   -+---------+---------+---------+-----
Pooled StDev =    4,089           0,0       6,0      12,0      18,0

Tukey's pairwise comparisons

    Family error rate = 0,0500
Individual error rate = 0,0102

Critical value = 5,67

Intervals for (column level mean) - (row level mean)

          CHILI PE    CHLORINE    CLOVE 48    RUE 48H

CHLORINE     -20,121
              12,671

CLOVE 48     -15,916     -12,191
              16,876      20,601

RUE 48H      -16,156     -12,431     -16,636
```

Figure 75. ANOVA analysis of Salicylic Acid production versus treatments applied: Chili Pepper, Chlorine, Clove, Rue and Water, 48 hours after.

Box plot analysis confirmed that treatments behaved equally, as all the boxes overlaped each other. Finally, Chlorine treatment had greater variability and water had less variability. Figure 76.

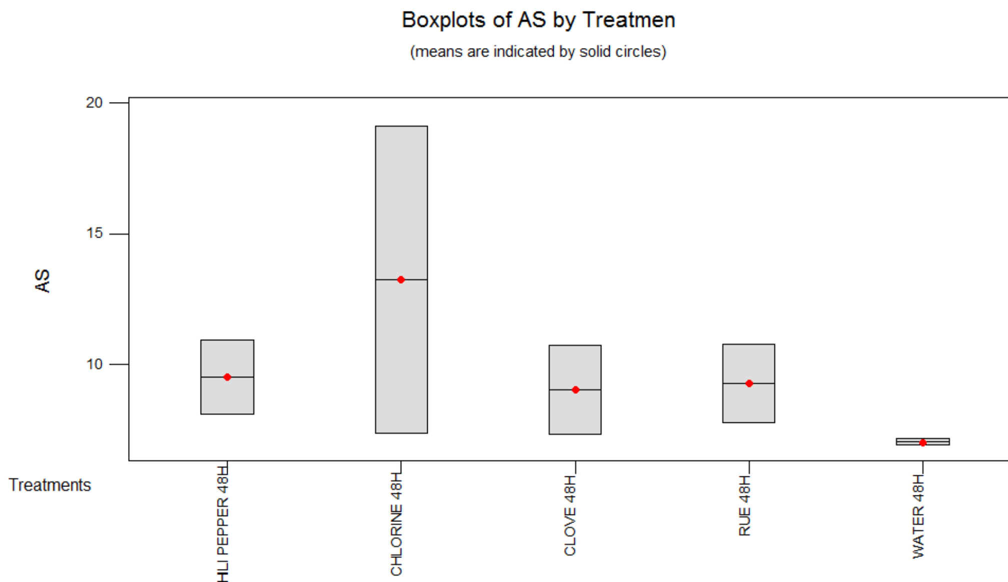


Figure 76. Box Chart of Treatments applied versus Salicylic Acid production, 48 hours after.

5.5 MINIMUM INHIBITORY CONCENTRATION MIC ASSAY RESULTS

Results for all samples evidenced that only pure extracts used as spraying treatments on tomato plants, were capable of inhibiting germ tubes formation in Oidium spores. As extracts were further double diluted, their action decreased significantly and hyphae proliferation was observed. Cell viability indicator Alamar Blue demonstrated that spores were viable depicted by a pink coloration developed in the media, while for negative control the dye remained blue. Figures 77 and 78.

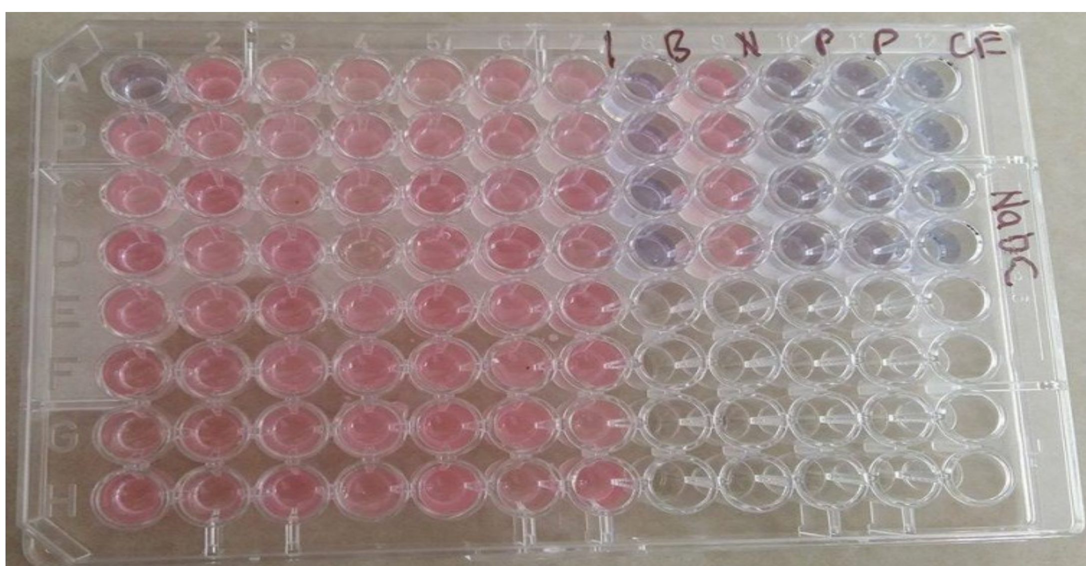


Figure 77. Cell viability assay in 96-well microplate. From left to right: line 1 to line 7 mixture of the substance, spores, cultivation broth. Line 8 blank, line 9 and line 10 negative control, line 11 and line 12 positive control. Assay for NaDC.

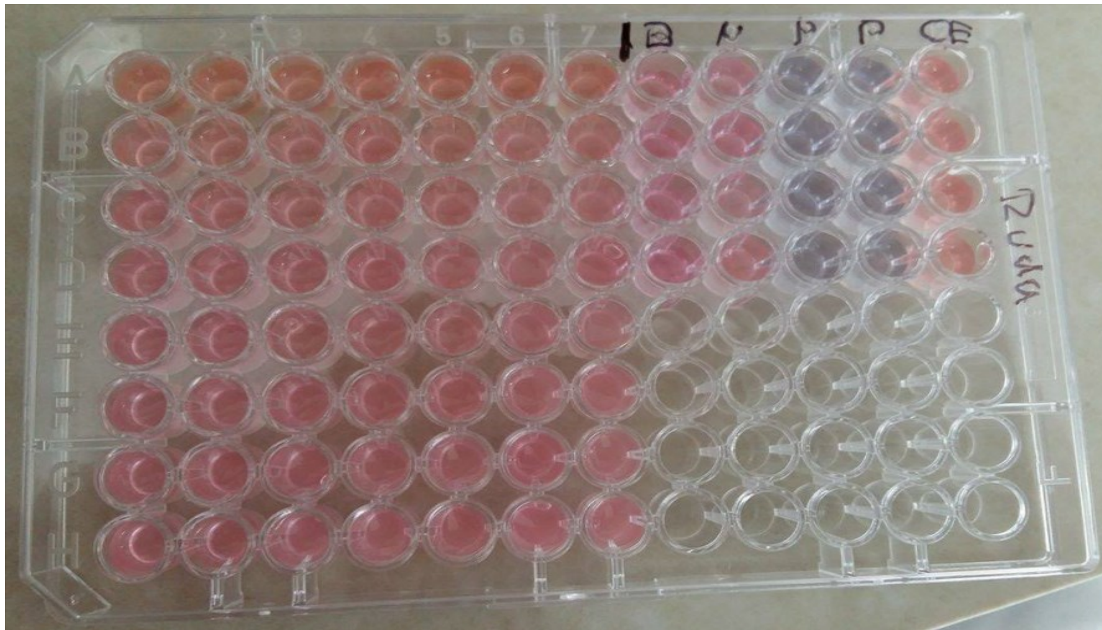


Figure 78. Cell viability assay in 96-well microplate. From left to right: line 1 to line 7 mixture of the substance, spores, cultivation broth. Line 8 blank, line 9 and line 10 negative control, line 11 and line 12 positive control. Assay for Rue.

5.6 CALLOSE DETERMINATION ASSAY RESULTS

Results of callose foldchange 24 and 48 hours after treatments were applied on tomato plants are presented in Figure 79.

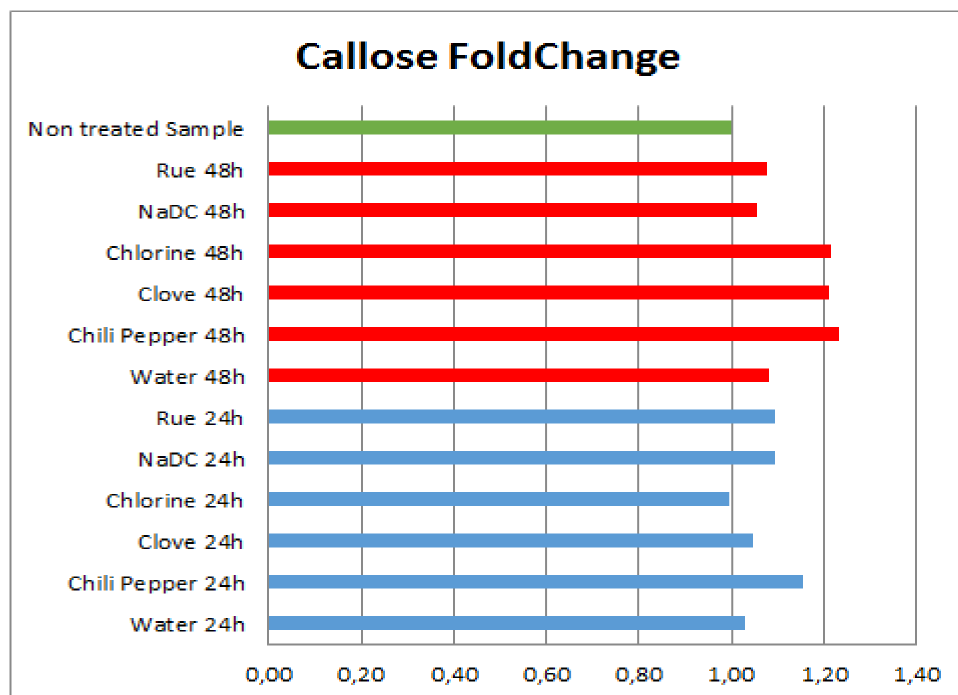


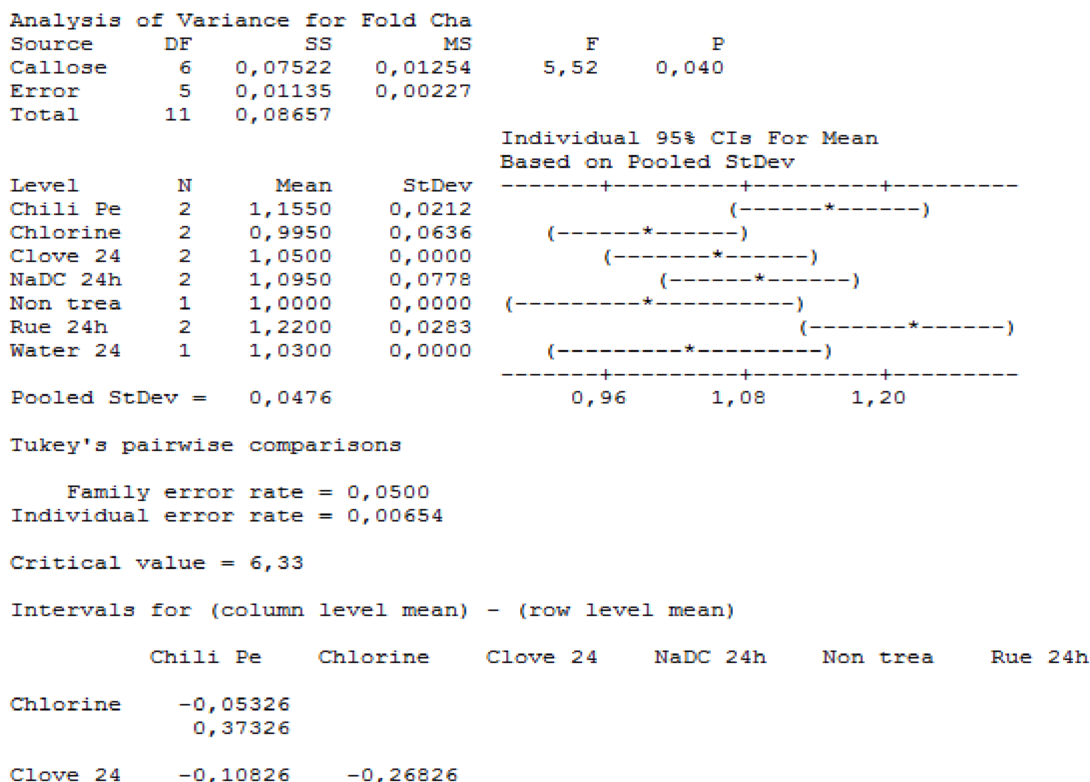
Figure 79. Callose FoldChange results after 24 and 48 hours.

Results at 24 hours evidenced that chili pepper treatment provided higher foldchange values followed by rue and clove. NaDC positive control was superior to water negative control.

48 hours values were higher for treatments with chili pepper, clove and chlorine, while for water remained the same as for 24 hours, similar to untreated sample.

To determine which treatment activated highest callose production a Tukey's test was performed with alpha = 0.05. The analysis showed that treatment with rue yielded more callose production; while Chili, Chlorine and Clove had similar behaviors. Water treatments produced less callose.

5.6.1 Statistical Analysis for Callose Determination Assay at 24 hours



One-way ANOVA: Fold Change versus Callose 24h

```
Analysis of Variance for Fold Cha
Source      DF        SS        MS        F        P
Callose      6   0,07522   0,01254     5,52    0,040
Error        5   0,01135   0,00227
Total       11   0,08657
                                   Individual 95% CIs For Mean
                                   Based on Pooled StDev
Level        N      Mean     StDev  -------+---------+---------+---------
Chili Pe     2    1,1550    0,0212                  (------*------)
Chlorine     2    0,9950    0,0636     (------*------)
Clove 24     2    1,0500    0,0000         (-------*------)
NaDC 24h     2    1,0950    0,0778             (------*------)
Non trea     1    1,0000    0,0000  (----------*----------)
Rue 24h      2    1,2200    0,0283                       (-------*------)
Water 24     1    1,0300    0,0000     (----------*---------)
                                    -------+---------+---------+---------
Pooled StDev =    0,0476                 0,96      1,08      1,20

Tukey's pairwise comparisons

    Family error rate = 0,0500
Individual error rate = 0,00654

Critical value = 6,33

Intervals for (column level mean) - (row level mean)

          Chili Pe    Chlorine    Clove 24    NaDC 24h    Non trea    Rue 24h

Chlorine    -0,05326
             0,37326

Clove 24    -0,10826    -0,26826
```

Figure 80. ANOVA analysis of Callose production versus treatments applied: Chili Pepper, Chlorine, Clove, Rue and Water, 24 hours after.

After performing ANOVA analysis, Figure 80, box plot confirmed that more callose production was obtained with rue extract treatment, without overlapping other

treatments. The ones that produce less callose were chlorine and untreated sample. Chili Pepper and Sodium Deoxicholate had callose production similar to treatments with Clove and Water. Figure 81.

Chlorine and Sodium Deoxicholate NaDC had also greater variability.

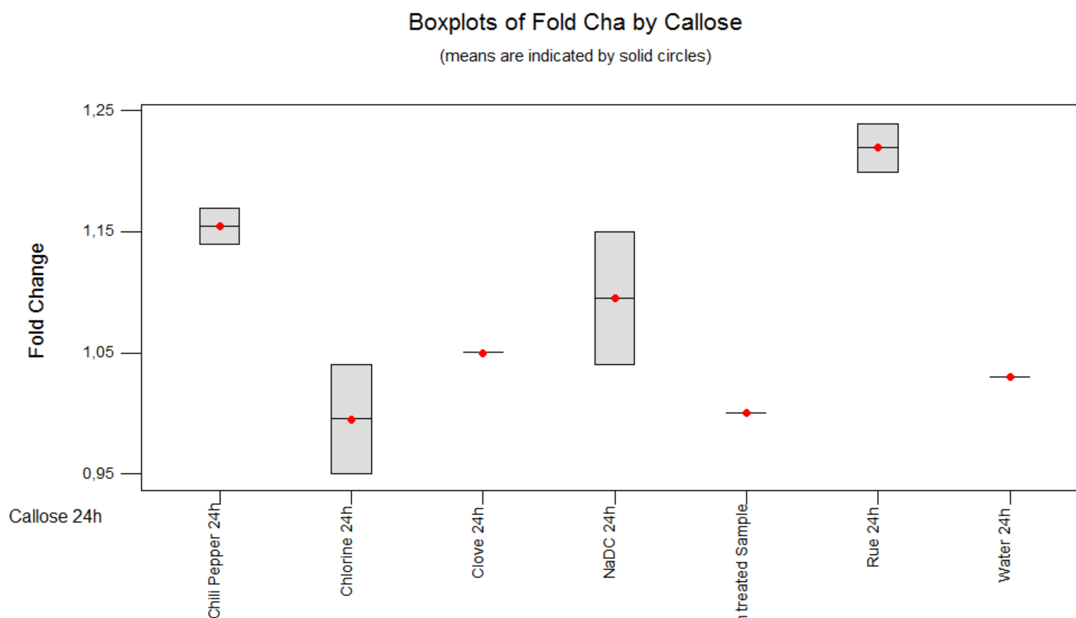


Figure 81. Box Chart of Treatments applied versus Callose production, 24 hours after.

5.6.2 Statistical Analysis for Callose Determination Assay at 48 hours

Since alpha and p values were very close, a Tukey's test was performed to determine if there was significant difference between these data. With Tukey's test alpha = 0.05, two groups that behaved differently were generated. Those corresponding to the first group, Chlorine, Chili Pepper and Clove; and in the second group Sodium Deoxicholate NaDC, Rue and Water. Figure 82.

Boxplots of Fold Cha by Callose

Normplot of Residuals for Fold Cha

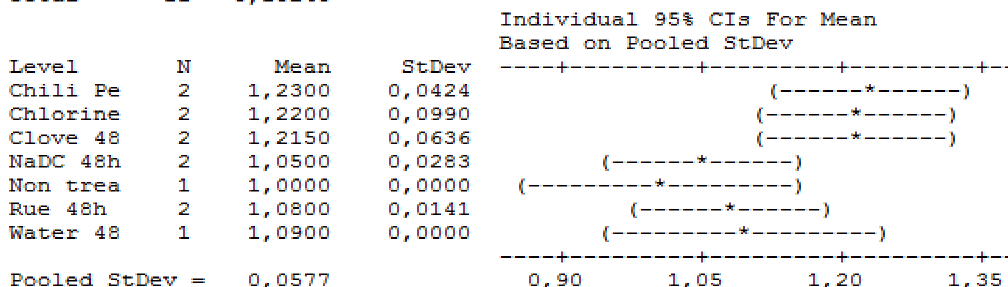
Saving file as: C:\Users\DRA. INES MALO\Desktop\MINITAB.MPJ as Callose 24h.MPJ

Results for: Worksheet 5

One-way ANOVA: Fold Change versus Callose 48h

Analysis of Variance for Fold Cha

Source	DF	SS	MS	F	P
Callose	6	0,08575	0,01429	4,29	0,066
Error	5	0,01665	0,00333		
Total	11	0,10240			



Individual 95% CIs For Mean Based on Pooled StDev

Level	N	Mean	StDev
Chili Pe	2	1,2300	0,0424
Chlorine	2	1,2200	0,0990
Clove 48	2	1,2150	0,0636
NaDC 48h	2	1,0500	0,0283
Non trea	1	1,0000	0,0000
Rue 48h	2	1,0800	0,0141
Water 48	1	1,0900	0,0000

Pooled StDev = 0,0577 — 0,90 1,05 1,20 1,35

Tukey's pairwise comparisons

Family error rate = 0,0500
Individual error rate = 0,00654

Figure 82. ANOVA analysis of Callose production versus treatments applied: Chili Pepper, Chlorine, Clove, Rue and Water, 48 hours after

Box chart confirms what Tukey's test released. In addition, the first group had a greater variability while the second group had a minimal variability. Figure 83.

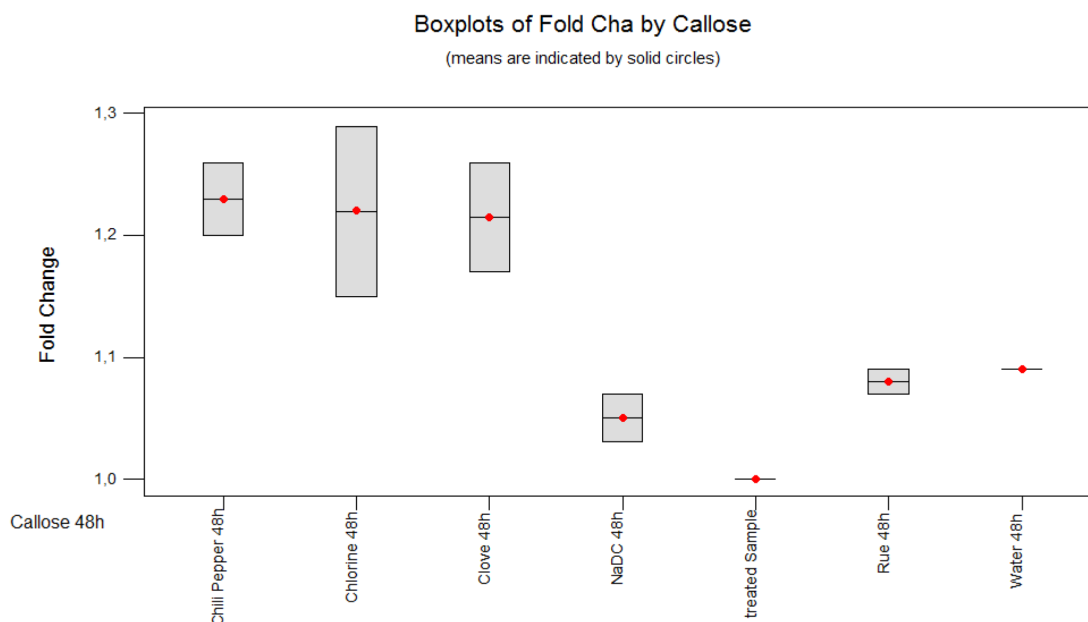


Figure 83. Box Chart of Treatments applied versus Callose production, 48 hours after.

CHAPTER 6

CONCLUSIONS

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6. CONCLUSIONS

The priming effect of some substances on plants defenses can constitute a strategy to reduce the use of agrochemicals. These substances known as elicitors have various chemical composition and could activate different types of defense responses in plants, allowing these to be faster or more robust, for example inducing callose deposition and activating salicylic acid/jasmonic acid SA/JA signaling pathways. (Aranega-Bou, 2014) It is projected that plant biostimulants market will increase for the coming years. (Calvo & al., 2014)

The results obtained in the present work confirm those by new studies, besides the ones already mentioned throughout this document, for example the use of winery by-products containing polyphenols and anthocyanins can induce defense genes in tobacco plants. (Benouaret & al., 2014), deoxycholic acid (DCA) is a promising molecule able to trigger defenses in plants, through the accumulation of reactive oxygen species and by activating the salicylic acid/jasmonic acid SA/JA signaling pathways. (Zarattini & al., 2016) Furthermore, deoxycholate can reduce the proliferation in bacterial infections with Erwinia amylovora and Pseudomonas syringae, constituting an effective method to protect crops from diseases. (Zarattini & al., 2016)

In the present work, the search for natural extracts capable of stimulate defense mechanisms was carried out on tomato plants Solanum lycopersicum L. aiming to help the low production of this species in regions where traditionally these crops were grown but have been abandoned for the presence of pests and diseases. These stresses caused a decrease in productivity bringing economic loss and forcing growers to replace tomato with other products with lower risk.

While efforts to find substances that are environmentally friendly and effective have been implemented in many countries, the possibility to find natural extract is a contribution that could alleviate the situation in affected areas, using wild species occurring naturally and which are known for their ancestral use but were left aside since the advent of the green revolution.

The results obtained in tomato plants can serve as a model to determine the effectiveness of natural extracts in other crops, promoting the rational use of substances for the benefit of both the environment and consumers.

Although the use of plants to prevent infestations by insects and/or diseases has been known and used for a long time as part of sustainable agriculture principles, as is the use of companion planting a passive mechanism that helps to maintain pests far from crops of interest, the interaction mechanisms involved have not been fully understood and are subject of constant research. (Kuepper & Dodson, 2016)

As in the case of agrochemicals, natural extracts can be sprayed as an intensive method for pests and disease treatment. The present investigation encourages the use and suitability of natural substances able to activate plant defense mechanisms. In addition, the preparation methods for sprayed solutions are safe and easy to carry out by non-expert hands and do not represent a problem or threat to the environment. The effectivity of the applied treatments to activate plants natural defenses was evaluated by molecular biology, histochemical, analytical chemistry and leaf infiltration tests.

The activation of plant defenses induced by natural extracts is based on a complex interaction between different plant hormones, signaling molecules and regulatory pathways. A similar situation has been observed in other species treated with active chlorine. (Zarattini & al., The use of ECAS in plant protection: a green and efficient antimicrobial approach that primes selected defense genes, 2015) It is well known that distinct levels of pathogen defenses are elicited by different and overlapping pathways (Conrath, 2009) (Dangl & al., 2013) for this reason it is not surprising that different plant extracts activate plant defenses in different manners.

The effect of priming plant natural defenses was demonstrated when natural extracts prepared with chili pepper, rue and clove were applied to tomato plants, resulting in an increased level of PR1 mRNA even up to 48 hours after the treatments; in a similar way to the levels found in positive controls. In the case of clove extract, it also increased the mRNA level of PR2 glucanase that protects plants against biotic stress. The accumulation of PR3, chitinase, was observed throughout the experiment, which may lead to disease resistance in plants and systemic acquired resistance SAR. Therefore, plant extracts used in the present

study appear able to activate and work both on salicylic acid SA and jasmonic acid JA signaling pathways. In Arabidopsis plants defense gene induction has been reported after the use of deoxycholic acid. DCA triggered defense activation, the production of significant amounts of reactive oxidative species ROS and the induction of defense genes like WRKY18, 33, 40, 46, 70, PR1, upregulation of 563 genes and downregulation of 47 genes as revealed by microarray analysis. (Zarattini & al., 2016)

Further indications on the effect of the natural extracts came from infiltration experiments. Both tobacco and tomato leaves showed necrosis in all the infiltration assays 1-2 days after the test, although clove extract was the best by comparing the necrosis areas. Similar results have been found in infiltration experiments with water extracts of winery by-products (red, white and seed grape extracts) inducing hypersensitive response in tobacco leaves and also stimulating PR1, PR2 and PR3 gene expression. (Benouaret & al., 2014)

The activity of natural extracts is additionally influenced by the type of solvent used during the extraction process. (Chang-Geun & al., 2011) Natural extracts, a mixture of active and non-active compounds, were aqueous except clove that was extracted with 70% ethanol and then diluted in water before spraying. Only undiluted extracts could stop fungus development in minimum inhibitory concentration MIC assays against Oidium spores. Studies on ethnobotanical uses of different plant species as in vitro antifungal solutions revealed that the pathogen growth inhibition strongly depends on the extracts concentration. (Diaz & al., 2011)

Callose determination by fluorometric assay showed that chili pepper and wild rue natural extracts induce callose accumulation 24 hours after treatment, at levels similar to those obtained by DCA 200 μM, while after 48 hours chili pepper and clove induced higher levels compared to DCA application. Similar results were obtained in Arabidopsis where a treatment with 200 μM DCA also induced callose deposition 24 h after treatment, as compared to mock-treated leaves. Callose depositions constitute a defense mechanism enhancing plant cell protection in different situations (Xiong-Yang & Jae-Yean, 2009), in particular callose deposits in response to pathogen attack. (Ellinger & Voigt, 2014)

The use of electrochemically activated solutions ECAS, containing hypochlorous acid, has proven to be effective both on tobacco plants and apple orchard trees,

by exerting an antimicrobial and antifungal effect as well as priming plant defenses. ECAS was shown to produce a tenfold increase in gene expression of PR1a, PR2 and PR17 six hours after treatment, the overexpression remained high at later stages too. Even plants treated a second time 14 days after the first treatment showed high levels of induction for PR1a and PR2 (up to 1000 fold change) (Zarattini & al., The use of ECAS in plant protection: a green and efficient antimicrobial approach that primes selected defense genes, 2015)

Similarly clove, pepper and rue extracts re-applied to tomato plants 15 days after a first treatment induced defense genes expression even if the levels were not as high and significant as the ones in the first application.

Through the screening of chemical libraries, other substances like diuretics have been found to activate plant defenses. Though they prime plant immunity they do not function as SA analogs, but they are able to confer disease tolerance in plants by an unknown mechanism. (Noutoshi & al., Diuretics Prime Plant Immunity in Arabidopsis thaliana, 2012) ImprimatinC1 is a weak SA analogue and its metabolites are partial agonist to SA, activating defense-related genes in Arabidopsis. 100μM of imprimatinC1 was able to increase the expression levels of PR1 in seedlings 24 hours after treatment. ((Noutoshi & al., ImprimatinC1 a novel plant immune-priming compound, functions as a partial agonist of salicylic acid, 2012) (Noutoshi & al., Novel Plant Immune-Priming Compounds Identified via High-Throughput Chemical Screening Target Salicylic Acid Glucosyltransferases in Arabidopsis, 2012) The data collected in this study reveal that natural extracts, obtained from chili pepper, wild rue and clove, when applied to tomato plants, are able to induce an increased transcription of defense and regulatory genes thus showing a priming effect. These plant extracts appear also able to activate both SA dependent and independent signaling pathways and to induce necrotic priming-related symptoms when infiltrated directly into leaves.

These easy-to-make extracts are derived from plant material that can be easily found, having low cost and being known for their properties. These extracts combine several interesting features (antimicrobial effects, resistance-induction, inexistent toxicity for the environment) as revealed by molecular and other studies. They could therefore be used as phytoprotective agriculture agents in the fight against pathogens.

After the development of the present investigation, it was verified that substances ancestrally used for pests and disease control in plants can effectively prime plant defenses, but these have been replaced because agrochemicals represent a faster system to obtain results (pest and disease elimination) compared to the use of plant-derived materials.

In future investigations, other plants extracts or the combination of those should be taken into consideration as a source of substances that can activate defense systems in a more effective way.

The limitations of the present work refer to the possibility of carrying out a field test with the preparations during the whole life time of a tomato plot, from its development as seedlings until the fruit harvest. Considering that the experimental part was done in a growth chamber, it was not possible to reach the complete development stage of tomato plants. In the future will be interesting to perform more investigations to integrate the laboratory results with the field tests, comparing fruit size and actual production yield to the traditional use of agrochemicals.

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ANNEXES

ANNEX A

ANNUAL REPORT OF ACTIVITIES 2014

Università degli Studi di Ferrara

ATTIVITA' DEI DOTTORANDI

RELAZIONE DI PREVENTIVO/ CONSUNTIVO

ANNO SOLARE 2014

In fase di **preventivo** e' sufficiente compilare i campi con l'asterisco e inviare la scheda al proprio coordinatore.
In fase di consuntivo:
1) ciascun DOTTORANDO compilerà la scheda per quanto di propria pertinenza la invierà tramite e-mail al Coordinatore
2) il COORDINATORE dovrà far pervenire tramite e-mail (dal proprio indirizzo di posta elettronica) all'Ufficio Post-Laurea (**dottorato@unife.it**) **ENTRO IL 9 DICEMBRE di ogni anno** tutte le SCHEDE debitamente compilate.

DOTTORATO DI RICERCA E CICLO*:

anno di corso: 1 ☑ 2 ☐ 3 ☐ proroga ☐

DOTTORANDO (nome e cognome)*: Inés Patricia Malo Cevallos
luogo e data di nascita Quito, November 11/1971
anno conseguimento laurea 1995
sede conseguimento laurea Cuenca – Ecuador-South America

TUTORE INTERNO *: Dr. Giovanni Bernacchia
EVENTUALE TUTORE ESTERNO*:

POSIZIONE (borsista ministeriale, borsista di altro ente, assegnista di ricerca, altra borsa, altro)*
Altro.

DESCRIZIONE DELL'ATTIVITA' DIDATTICA E SCIENTIFICA

NB: per il preventivo compilare almeno i campi contrassegnati dall'asterisco

ATTIVITA' DIDATTICA

Attività didattica trasversale (*)

TITOLO DELL'ATTIVITÀ'	crediti
1 Italian Course at Societá Dante Alighieri, Cuenca, level 4, 120 hours.	
2.. Human Development and University CDHU, Polytechnic Salesian University, 80 hours.	
3.. Scientific Writing, Polytechnic Salesian University, 60 hours	
4.. Teachers Blog, Polytechnic Salesian University, 1 hour.	
5.. Occupational Health, Polytechnic Salesian University, 1 hour.	
6.. Accessibility in virtual environments, Polytechnic Salesian University, 1 hour.	

Attività didattica di macroarea (*)

TITOLO DELL'ATTIVITÀ'	crediti
1 Upgrade in Biotechnology, 200 hours, University of Guayaquil	
2..	

Frequenza di insegnamenti (*)

TITOLO DELL' INSEGNAMENTO, CORSO DI STUDIO E SEDE	crediti
1. Molecular Biology, 40 hours, University of Azuay	
2..	

Frequenza di seminari ai quali il Dottorando partecipa come uditore (max 5)

TITOLO DEL SEMINARIO	DOCENTE	SEDE E DATA	crediti

1. Waste Management in the Oil Industry	Fernando Morales	Polytechnic Salesian University, 18-21 March 2014.	
2…Statistics	Pablo Arevalo Moscoso	Polytechnic Salesian University, July 2014.	

Seminari tenuti dal Dottorando (max 3)

TITOLO DEL SEMINARIO	SEDE E DATA	crediti
1 Activation of defense genes in tomato plants, Lycopersicum esculentum L., through the application of chemical and natural substances	Portoviejo - Ecuador, october the 16th 2014.	
2..		

Attività didattica svolta dal dottorando nei corsi di studio dell'Università di Ferrara

Insegnamento, corso di studio e docente	Tipo di attività (lezioni, esercitazioni, assistenza in laboratorio)	Numero di ore	crediti
1.			
2...			

Altre attività didattiche

Tipologia	crediti
1. Biology I, Polytechnic Salesian University, 64 hours	
2.. Biology II, Polytechnic Salesian University, 64 hours	
3.. General Biotechnology, Polytechnic Salesian University, 48 hours	
4.. Introduction to Microbiology, Polytechnic Salesian University, 64 hours	
5.. Industrial Microbiology, Polytechnic Salesian University, 64 hours	
6.. Principles of Genetics, Polytechnic Salesian University, 48 hours	
7.. Analytical Chemistry, Polytechnic Salesian University, 64 hours	
8.. Processing of Cosmetics, Polytechnic Salesian University, 32 hours	

ATTIVITA' SCIENTIFICA

Stage svolti presso strutture all'estero

SEDE	PERIODO DELLO STAGE (dal ……. al ………)	ATTIVITA' SVOLTA	crediti
1.			
2.			

Stage svolti presso strutture in Italia

SEDE	PERIODO DELLO STAGE (dal ……. al ………)	ATTIVITA' SVOLTA	crediti
1.			
2.			

Corsi e Scuole di Formazione

Corso/Scuola di formazione	Sede e durata	crediti
1. Master in Biotechnology Final Exam	University of Guayaquil	
2.		

Convegno-Workshop, ecc.	Sede	Presentazione di una relazione (barrare la voce che interessa)	crediti
1. II Ecuadorian Congress of the Polytechnic University Net for Research and Graduate	Loja - Ecuador	SI **NO**	

2. 4th International Symposium of Multidisciplinary Research, 1st International Congress "The Research and the Good Living"	Portoviejo - Ecuador	**SI**	NO	

Partecipazione a Convegni, Workshop, Giornate di studio, ecc.

Pubblicazioni (solo articoli già apparsi o in corso di pubblicazione su una rivista/atti/libro, max 3)

Titolo	Autori (nell'ordine)	Titolo della Rivista o degli Atti del Convegno e pagine
1. Activation of defense genes in tomato plants, *Lycopersicum esculentum L.*, through the application of chemical and natural substances	Inés Malo	Memories Program and Abstracts, page 134, 4th International Symposium of Multidisciplinary Research, 1st International Congress "The Research and the Good Living"
2. Effects of the application of substances on three WRKY genes in tomato plants *Lycopersicum esculentum L.*	Inés Malo	Memories Program and Abstracts II Ecuadorian Congress of the Polytechnic University Net for Research and Graduate
3.		

Partecipazione a progetti di ricerca correlati a quello di Dottorato, max 3

Ente finanziatore	Titolo del progetto di ricerca	Responsabile del progetto di ricerca e mesi uomo richiesti al dottorando
1. Polytechnic Salesian University	"Study of the PR defense genes by the application of pepper extract (Capsicum anuum) solimanillo in a tomato crop (Lycopersicum esculentum)"	Pablo Arevalo Moscoso, 1 year, 2014
2. Polytechnic Salesian University	"Study of the PR defense genes by the application of activated water in a tomato crop (Lycopersicum esculentum)"	Pablo Arevalo Moscoso, 1 year, 2013
3.		

(**Descrizione dell'attività di ricerca svolta e dei risultati ottenuti: massimo 1000 caratteri)**

Determine the activation of defense genes PR1a, PR1b, basic Glucanase and acidic chitinase in tomato plants Lycopersicon esculentum L. The plants were grown from seed under controlled conditions in a growth chamber. Extracts were prepared with rocoto pepper fruits purchased in local markets, cut and placed in water for 24 hours. After filtering the pH of the solution was adjusted and applied to plants. Leaf samples were harvested at different timepoints for RNA extraction, cDNA synthesis and amplification by qPCR. The gene activation observed with the pepper extract was compared with the one obtained with diluted bleach (positive control) and with water (negative control).
In the next future we will continue the study testing other natural substances and varying concentrations of the extracts. Also make the Minimum Inhibitory Concentration of natural substances in relation to the fungus Oidium, a pathogen commonly presented in tomato crops.

crediti ………………..

Altro (incluso attività continuative extra-dottorato svolte nell'anno) **crediti** …………….

PARTE DA COMPILARE A CURA DEL COORDINATORE

Per i dottorandi del PRIMO ANNO

Il dottorando ha sostenuto la prova di verifica in data ______________________ riportando il seguente giudizio:

☐ **NON IDONEO: non viene pertanto ammesso al proseguimento del dottorato di ricerca e incorre nella decadenza**

☐ **IDONEO: ammesso al secondo anno**

solo per i seguenti corsi di dottorato è previsto al termine del primo anno di corso il rilascio del Master Scientifico Culturale:
- FISICA: MSC in Fisica;
- MATEMATICA E INFORMATICA: MSC in Matematica e informatica;
- SCIENZE BIOMEDICHE: MSC in
Neurofisiologia Clinica, Principi Tecnici ed Applicazioni Cliniche
Epilettologia
Emodinamica del sistema venoso
- SCIENZE DELL'INGEGNERIA: MSC in Scienze dell'ingegneria

☐ **IL DOTTORANDO ha conseguito il M.S.C. in ____ con la seguente votazione (in trentesimi) ___/30 (_______________/trenta)**

Per i dottorandi del SECONDO ANNO

Il dottorando ha presentato la relazione sulle attività svolte al Collegio Docenti in data __________________ riportando il seguente giudizio:

☐ **NON IDONEO: non viene pertanto ammesso al proseguimento del dottorato di ricerca e incorre nella decadenza**

☐ **IDONEO: ammesso al terzo anno**

Per i dottorandi del TERZO ANNO e dottorandi in PROROGA

VALUTAZIONE DEL COLLEGIO DEI DOCENTI SULLA TESI

____
____
____
____
____

Il dottorando ha presentato la relazione sulle attività svolte al collegio docenti in data __________________ riportando il seguente giudizio:

☐ **NON IDONEO: non viene pertanto ammesso all'esame finale e incorre nella decadenza**

☐ **IDONEO: ammesso all'esame finale**

☐ **Il Collegio Docenti in data __________________, ha autorizzato la redazione della tesi in lingua __________________________.**

☐ **Il Collegio Docenti in data __________________, valutate le motivazioni presentate dal dottorando, propone al Rettore di concedere l'ammissione al**

☐ **PROROGA di un anno per la presentazione della tesi**
(previa presentazione della relativa domanda da parte del dottorando entro i termini previsti dal regolamento di Dottorato)

☐ **Il Collegio Docenti in data ____________________, ha proposto il rilascio della certificazione aggiuntiva di DOCTOR EUROPAEUS**

Data _________________________ IL COORDINATORE DEL DOTTORATO

....

Università degli Studi di Ferrara

ATTIVITA' DEI DOTTORANDI
RELAZIONE DI PREVENTIVO/ CONSUNTIVO
ANNO SOLARE 2015

In fase di preventivo **e' sufficiente compilare i campi con l'asterisco e inviare la scheda al proprio coordinatore.**
In fase di consuntivo:
1) ciascun DOTTORANDO compilerà la scheda per quanto di propria pertinenza la invierà tramite e-mail al Coordinatore
2) il COORDINATORE dovrà far pervenire tramite e-mail (dal proprio indirizzo di posta elettronica) all'Ufficio Post-Laurea (dottorato@unife.it**)** ENTRO IL 9 DICEMBRE di ogni anno **tutte le SCHEDE debitamente compilate.**

DOTTORATO DI RICERCA E CICLO*:

anno di corso: 1 ☐ 2 ☑ 3 ☐ proroga ☐

DOTTORANDO (nome e cognome)*: Inés Patricia Malo Cevallos
luogo e data di nascita: Quito, november 11 / 1971.
anno conseguimento laurea: 1995
sede conseguimento laurea: Cuenca – Ecuador-Sud America

TUTORE INTERNO *: Dr. Giovanni Bernacchia
EVENTUALE TUTORE ESTERNO*:

POSIZIONE (borsista ministeriale, borsista di altro ente, assegnista di ricerca, altra borsa, altro)*
Altro.

DESCRIZIONE DELL'ATTIVITA' DIDATTICA E SCIENTIFICA
NB: per il preventivo compilare almeno i campi contrassegnati dall'asterisco
ATTIVITA' DIDATTICA
Attività didattica trasversale (*)

TITOLO DELL'ATTIVITÀ'	crediti
1. Scientific Writing II, Universidad Politécnica Salesiana, 60 hours.	
2. Investigation, Cientific Production and University Press, 24 hours.	
3. Gestione della ricerca, della conoscenza dei sistemi di ricerca e di finanziamento. Prof. Silvano Capitani.	

Attività didattica di macroarea (*)

TITOLO DELL'ATTIVITÀ'	crediti
1. Theoretical and practical course of genetic transformation: new concepts and tools, 45 hours.	
2.	

Frequenza di insegnamenti (*)

TITOLO DELL' INSEGNAMENTO, CORSO DI STUDIO E SEDE	crediti
1. English exam, approved group D. English Classes. Alisson Mary Milne.	
2. I Heart Stats: Learning to Love Statistics. University of Notre Dame. EdX Course.	

Frequenza di seminari ai quali il Dottorando partecipa come uditore (max 5)

TITOLO DEL SEMINARIO	DOCENTE	SEDE E DATA	crediti

Simulazione di Logical Framework, Biotrade and Value Chain Analysis.	Matteo Radice.	Universitá degli Studi di Ferrara 17/4/2015	
Tracing Human Migration from a bacterial perspective.	Dr. Yoshan Moodley.	Universitá degli Studi di Ferrara 21/4/2015	
L´occhio di Galileo. Una rivoluzione nella fisiología en ella filosofía dei sensi.	Marco Picolino.	Universitá degli Studi di Ferrara 28/4/2015	
Le Biotecnologie Verdi come opportunitá di business per l´avanzamento economico in Europa.	Dott. Franco Fornasari.	Universitá degli Studi di Ferrara 5/5/2015	
Dalla ricerca all´impresa quali percorsi perla commercializzazione dei risultati.	Dott. Franco Fornasan	Universitá degli Studi di Ferrara 7/5/2015	

Seminari tenuti dal Dottorando (max 3)

TITOLO DEL SEMINARIO	SEDE E DATA	crediti
Genetic modification in plants, GMO.	Universidad Politécnica Salesiana, Cuenca, 10/7/2015	

Attività didattica svolta dal dottorando nei corsi di studio dell'Università di Ferrara

Insegnamento, corso di studio e docente	Tipo di attività (lezioni, esercitazioni, assistenza in laboratorio)	Numero di ore	Crediti

Altre attività didattiche

Tipologia	crediti
1. Environmental Biotechnology, Universidad Politécnica Salesiana, 64 hours.	
2. Introduction to Microbiology, Universidad Politécnica Salesiana, 64 hours.	
3. Principles of Genetics, Universidad Politécnica Salesiana, 64 hours.	
4. Bioinformatics, 16 hours.	
5. Natural resources research, 48 hours.	
6. Introduction to laboratory practice, 16 hours.	
7. Introduction to Biology, 20 hours.	
8. Introduction to Chemistry, 20 hours.	

ATTIVITA' SCIENTIFICA

Stage svolti presso strutture all'estero

SEDE	PERIODO DELLO STAGE (dal ……. al ………)	ATTIVITA' SVOLTA	crediti
1. Theoretical and practical course of genetic transformation: new concepts and tools, 45 hours.	June 29 to July 3	Transient transformation of Tobacco plants mediated by Agrobacterium tumefaciens with GUS Gene.	
2.			

Stage svolti presso strutture in Italia FERRARA

SEDE	PERIODO DELLO STAGE (dal ……. al ………)	ATTIVITA' SVOLTA	crediti
Universitá degli Studi di Ferrara	February2015/May2015	GUS Assay in tomato plants treated with pepper extract.	

Universitá degli Studi di Ferrara	February2015/May2015	Culture of transgenic tomato and tobacco plants in the growing chamber.	
Universitá degli Studi di Ferrara	February2015/May2015	Bacterial DNA extraction.	
Universitá degli Studi di Ferrara	February2015/May2015	PCR analysis.	
Universitá degli Studi di Ferrara	February2015/May2015	Bacterial DNA purification.	
Universitá degli Studi di Ferrara	February2015/May2015	Analysis of the sequenced fragments with BLAST.	
Universitá degli Studi di Ferrara	February2015/May2015	Protein electroforesis.	
Universitá degli Studi di Ferrara	February2015/May2015	Use of the french press to disrupt cells.	
Universitá degli Studi di Ferrara	February2015/May2015	Use of thin layer chromatography for cholic acids.	

Corsi e Scuole di Formazione

Corso/Scuola di formazione	Sede e durata	crediti

Partecipazione a Convegni, Workshop, Giornate di studio, ecc.

Convegno-Workshop, ecc.	Sede	Presentazione di una relazione (barrare la voce che interessa)	crediti
Investigation, Cientific Production and University Press, 24 hours.	Cuenca-Ecuador	SI **NO**	

Pubblicazioni (solo articoli già apparsi o in corso di pubblicazione su una rivista/atti/libro, max 3)

Titolo	Autori (nell'ordine)	Titolo della Rivista o degli Atti del Convegno e pagine
ACTIVATION OF DEFENSE GENES IN TOMATO PLANTS, *Solanum lycopersicum L.*, THROUGH THE APPLICATION OF CHEMICAL AND NATURAL SUBSTANCES	Inés Malo[1], Giovanni Bernacchia[2], Morena De Bastiani[2], Pablo Arevalo[1].	"La Granja" (Cuenca – Ecuador)

Partecipazione a progetti di ricerca correlati a quello di Dottorato, max 3

Ente finanziatore	Titolo del progetto di ricerca	Responsabile del progetto di ricerca e mesi uomo richiesti al dottorando
1. Polytechnic Salesian University	"Study of the PR defense genes and WRKY genes by the application of pepper extract (Capsicum anuum) solimanillo in a tomato crop (Lycopersicum esculentum)"	Pablo Arevalo Moscoso, 1 year, 2015
2. Polytechnic Salesian University	"Study of the PR defense genes and WRKY genes by the application of activated water in a tomato crop (Lycopersicum esculentum)"	Pablo Arevalo Moscoso, 1 year, 2014

(**Descrizione dell'attività di ricerca svolta e dei risultati ottenuti: massimo 1000 caratteri)**

Determine the activation of defense genes PR1a, PR1b, basic Glucanase and acidic chitinase; transcription factors WRKY8 , WRKY23 , WRKY39 , in tomato plants *Lycopersicon esculentum L*. The plants were grown from seed under controlled conditions in a growth chamber. Rocoto pepper, ruta and clove were purchased in local markets. Rocoto pepper fruits were cut and placed in water for 24 hours, decoction for ruta extract, and ethanolic clove extract. After filtering the pH of the solution was adjusted and applied to plants. Leaf samples were harvested at different timepoints for RNA extraction, cDNA synthesis and amplification by qPCR. The gene activation observed with the pepper extract, ruta extract and ethanolic extract of clove, was

compared with the one obtained with diluted bleach (positive control) and with water (negative control).
Minimum Inhibitory Concentration of the natural substances in relation to the fungus Oidium, a pathogen commonly presented in tomato crops.

crediti ………………..

Altro (incluso attività continuative extra-dottorato svolte nell'anno)
…… …………
………
crediti …………….

Dr. Giovanni Bernacchia   Inés Malo Cevallos

PARTE DA COMPILARE A CURA DEL COORDINATORE

Per i dottorandi del PRIMO ANNO

Il dottorando ha sostenuto la prova di verifica in data _____________________ riportando il seguente giudizio:

☐ **NON IDONEO: non viene pertanto ammesso al proseguimento del dottorato di ricerca e incorre nella decadenza**

☐ **IDONEO: ammesso al secondo anno**

solo per i seguenti corsi di dottorato è previsto al termine del primo anno di corso il rilascio del Master Scientifico Culturale:
- FISICA: MSC in Fisica;
- MATEMATICA E INFORMATICA: MSC in Matematica e informatica;
- SCIENZE BIOMEDICHE: MSC in
Neurofisiologia Clinica, Principi Tecnici ed Applicazioni Cliniche
Epilettologia
Emodinamica del sistema venoso
- SCIENZE DELL'INGEGNERIA: MSC in Scienze dell'ingegneria

☐ **IL DOTTORANDO ha conseguito il M.S.C. in ____ con la seguente votazione (in trentesimi) ___/30 (_______________/trenta)**

Per i dottorandi del SECONDO ANNO

Il dottorando ha presentato la relazione sulle attività svolte al Collegio Docenti in data __________________ riportando il seguente giudizio:

☐ **NON IDONEO: non viene pertanto ammesso al proseguimento del dottorato di ricerca e incorre nella decadenza**

☐ **IDONEO: ammesso al terzo anno**

Per i dottorandi del TERZO ANNO e dottorandi in PROROGA

VALUTAZIONE DEL COLLEGIO DEI DOCENTI SULLA TESI

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Il dottorando ha presentato la relazione sulle attività svolte al collegio docenti in data __________________ riportando il seguente giudizio:

☐ **NON IDONEO: non viene pertanto ammesso all'esame finale e incorre nella decadenza**

☐ **IDONEO: ammesso all'esame finale**

☐ **Il Collegio Docenti in data __________________, ha autorizzato la redazione della tesi in lingua __________________________.**

☐ **Il Collegio Docenti in data __________________, valutate le motivazioni presentate dal dottorando, propone al Rettore di concedere l'ammissione al**

☐ **PROROGA di un anno per la presentazione della tesi**
(previa presentazione della relativa domanda da parte del dottorando entro i termini previsti dal regolamento di Dottorato)

☐ **Il Collegio Docenti in data ____________________, ha proposto il rilascio della certificazione aggiuntiva di DOCTOR EUROPAEUS**

Data _________________________ IL COORDINATORE DEL DOTTORATO

....

ANNEX C

ANNUAL REPORT OF ACTIVITIES 2016

Università degli Studi di Ferrara

ATTIVITA' DEI DOTTORANDI

RELAZIONE DI PREVENTIVO/ CONSUNTIVO

ANNO SOLARE 2016

In fase di preventivo **e' sufficiente compilare i campi con l'asterisco e inviare la scheda al proprio coordinatore.**
In fase di consuntivo:
1) ciascun DOTTORANDO compilerà la scheda per quanto di propria pertinenza la invierà tramite e-mail al Coordinatore
2) il COORDINATORE dovrà far pervenire tramite e-mail (dal proprio indirizzo di posta elettronica) all'Ufficio Post-Laurea (dottorato@unife.it) ENTRO IL 9 DICEMBRE di ogni anno **tutte le SCHEDE debitamente compilate.**

DOTTORATO DI RICERCA E CICLO*:

anno di corso: 1 ☐ 2 ☐ 3 ☑ proroga ☐

DOTTORANDO (nome e cognome)*: Inés Patricia Malo Cevallos
luogo e data di nascita: Quito, november 11 / 1971.
anno conseguimento laurea: 1995
sede conseguimento laurea: Universidad de Cuenca – Ecuador-Sud America

TUTORE INTERNO *: Dr. Giovanni Bernacchia
EVENTUALE TUTORE ESTERNO*:

POSIZIONE (borsista ministeriale, borsista di altro ente, assegnista di ricerca, altra borsa, altro)*
Altro.

DESCRIZIONE DELL'ATTIVITA' DIDATTICA E SCIENTIFICA
NB: per il preventivo compilare almeno i campi contrassegnati dall'asterisco
ATTIVITA' DIDATTICA

Attività didattica trasversale (*)

TITOLO DELL'ATTIVITÀ'	crediti
1. Litteracy, 20 hours.	
2. Learning Management, 20 hours.	
3. Thought Development, 20 hours.	

Attività didattica di macroarea (*)

TITOLO DELL'ATTIVITÀ'	crediti
1.	
2.	

Frequenza di insegnamenti (*)

TITOLO DELL' INSEGNAMENTO, CORSO DI STUDIO E SEDE	crediti
1. Italian course, approved level B2, Teacher Mimma Diaco.	

Frequenza di seminari ai quali il Dottorando partecipa come uditore (max 5)

TITOLO DEL SEMINARIO	DOCENTE	SEDE E DATA	crediti

Seminari tenuti dal Dottorando (max 3)

TITOLO DEL SEMINARIO	SEDE E DATA	crediti
Bioinformatics.	Universidad Politécnica Salesiana, Cuenca, 19/9/2016	

Attività didattica svolta dal dottorando nei corsi di studio dell'Università di Ferrara

Insegnamento, corso di studio e docente	Tipo di attività (lezioni, esercitazioni, assistenza in laboratorio)	Numero di ore	Crediti
Population Genetics, Dr. Guido Barbujani	Lezioni	26	
Forensic Genetics, Dr. Elizabetta Mamolini	Lezioni	12	

Altre attività didattiche

Tipologia	crediti
1. Environmental Biotechnology, Universidad Politécnica Salesiana, 64 hours.	
2. Introduction to Microbiology, Universidad Politécnica Salesiana, 64 hours.	
3. Principles of Genetics, Universidad Politécnica Salesiana, 64 hours.	
4. Bioinformatics, 16 hours.	
5. Waste Management, 16 hours.	
6. Introduction to laboratory practice, 16 hours.	
7. Good Agriculture Practices, 32 hours.	
8. Quantitative Analytical Chemistry, 64 hours.	

ATTIVITA' SCIENTIFICA

Stage svolti presso strutture all'estero

SEDE	PERIODO DELLO STAGE (dal ……. al ………)	ATTIVITA' SVOLTA	crediti
1.			
2.			

Stage svolti presso strutture in Italia FERRARA

SEDE	PERIODO DELLO STAGE (dal ……. al ………)	ATTIVITA' SVOLTA	crediti
Universitá degli Studi di Ferrara	February2016/April2016	Scientific paper search for the thesis.	
Universitá degli Studi di Ferrara	April 2016	Refreshing of Agrobacterium strains, antibiotic aliquot preparation.	

Corsi e Scuole di Formazione

Corso/Scuola di formazione	Sede e durata	crediti

Partecipazione a Convegni, Workshop, Giornate di studio, ecc.

Convegno-Workshop, ecc.	Sede	Presentazione di una relazione (barrare la voce che interessa)	crediti
1.		SI **NO**	

Pubblicazioni (solo articoli già apparsi o in corso di pubblicazione su una rivista/atti/libro, max 3)

Titolo	Autori (nell'ordine)	Titolo della Rivista o degli Atti del Convegno e pagine
ENERGY UTILIZATION OF PLASTIC WASTE OBTAINING LIQUID FUELS BY MEANS OF PYROLYSIS PROCESS	Myriam Mancheno* , Servio Astudillo, Pablo Arévalo, Inés Malo, Tania Naranjo, Johana Espinoza	"La Granja" (Cuenca – Ecuador) DOI:10.17163/lgr.n23.2016.06

Partecipazione a progetti di ricerca correlati a quello di Dottorato, max 3

Ente finanziatore	Titolo del progetto di ricerca	Responsabile del progetto di ricerca e mesi uomo richiesti al dottorando
1. Polytechnic Salesian University	"Study of the PR defense genes and WRKY genes by the application of pepper extract (Capsicum anuum) solimanillo in a tomato crop (Lycopersicum esculentum)"	Pablo Arevalo Moscoso, 1 year, 2016
2. Polytechnic Salesian University	"Study of the PR defense genes and WRKY genes by the application of activated water in a tomato crop (Lycopersicum esculentum)"	Pablo Arevalo Moscoso, 1 year, 2015
3. Polytechnic Salesian University	"Study of the PR defense genes and WRKY genes by the application of activated water in a tomato crop (Lycopersicum esculentum)"	Pablo Arevalo Moscoso, 1 year, 2014

(**Descrizione dell'attività di ricerca svolta e dei risultati ottenuti: massimo 1000 caratteri)**

Implementation of an HPLC methodology to quantify Salicylic Acid from tomato leaves. The plants were grown from seed under controlled conditions in a growth chamber. Rocoto pepper, ruta and clove were purchased in local markets. Rocoto pepper fruits were cut and placed in water for 24 hours, decoction for ruta extract, and ethanolic clove extract. After filtering the pH of the solution was adjusted and applied to plants. Leaf samples were harvested at different timepoints for Salicylic Acid extraction and quantification. The results obtained are similar to the ones determined by the gene activation observed with the pepper extract, ruta extract and ethanolic extract of clove, compared with the one obtained with diluted bleach (positive control) and with water (negative control).

Minimum Inhibitory Concentration of the natural substances in relation to the fungus Oidium, a pathogen commonly presented in tomato crops. Determination of callose foldchange in tomato plants 24 and 48 hours after the application of the treatments.

crediti ………………..

Altro (incluso attività continuative extra-dottorato svolte nell'anno)
……… …
……
crediti …………….

Dr. Giovanni Bernacchia   Inés Malo Cevallos

PARTE DA COMPILARE A CURA DEL COORDINATORE

<u>Per i dottorandi del PRIMO ANNO</u>

Il dottorando ha sostenuto la prova di verifica in data ____________________ riportando il seguente giudizio:

☐ **NON IDONEO: non viene pertanto ammesso al proseguimento del dottorato di ricerca e incorre nella decadenza**

☐ **IDONEO: ammesso al secondo anno**

<u>solo per i seguenti corsi di dottorato è previsto al termine del primo anno di corso il rilascio del Master Scientifico Culturale:</u>
- FISICA: MSC in Fisica;
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Neurofisiologia Clinica, Principi Tecnici ed Applicazioni Cliniche
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- SCIENZE DELL'INGEGNERIA: MSC in Scienze dell'ingegneria

☐ **IL DOTTORANDO ha conseguito il M.S.C. in ____ con la seguente votazione (in trentesimi) ___/30 (_______________/trenta)**

<u>Per i dottorandi del SECONDO ANNO</u>

Il dottorando ha presentato la relazione sulle attività svolte al Collegio Docenti in data __________________ riportando il seguente giudizio:

☐ **NON IDONEO: non viene pertanto ammesso al proseguimento del dottorato di ricerca e incorre nella decadenza**

☐ **IDONEO: ammesso al terzo anno**

<u>Per i dottorandi del TERZO ANNO e dottorandi in PROROGA</u>

VALUTAZIONE DEL COLLEGIO DEI DOCENTI SULLA TESI

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Il dottorando ha presentato la relazione sulle attività svolte al collegio docenti in data __________________ riportando il seguente giudizio:

☐ **NON IDONEO: non viene pertanto ammesso all'esame finale e incorre nella decadenza**

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☐ **Il Collegio Docenti in data __________________, ha autorizzato la redazione della tesi in lingua ___________________________.**

☐ **Il Collegio Docenti in data __________________, valutate le motivazioni presentate dal dottorando, propone al Rettore di concedere l'ammissione al**

☐ **PROROGA di un anno per la presentazione della tesi**
(previa presentazione della relativa domanda da parte del dottorando entro i termini previsti dal regolamento di Dottorato)

☐ **Il Collegio Docenti in data ____________________, ha proposto il rilascio della certificazione aggiuntiva di DOCTOR EUROPAEUS**

Data ________________________ IL COORDINATORE DEL DOTTORATO

....

ANNEX D

Poster presented at the II CONGRESO AUSENP held in the City of Loja Ecuador, October 2014.

"Efectos de la aplicación de sustancias sobre tres genes WRKY en plantas de tomate de mesa *Lycopersicum esculentum L*"

Malo Cevallos, I.[1], Arévalo Moscoso, P.[1], Bernacchia, G.[2]

[1] Dpto. Biotecnología. Universidad Politécnica Salesiana. Campus El Vecino. Calle Vieja 12-30 y Elia Liut
Tel.:(593) 72862213
[2] Dpto. Scienze della Vita e Biotecnologie. Università di Ferrara, Italia.
Tel. +39 0532 455784

[1] Autor para correspondencia: imalo@ups.edu.ec

Introducción

Lycopersicum esculentum L. es una planta cuyo cultivo en el país, tanto a cielo abierto como en invernadero, está sujeto a la utilización de agroquímicos, se ve afectada por una gran variedad de plagas y condiciones ambientales adversas (1). El cultivo y consumo de tomate de mesa se encuentra muy difundido en nuestro país debido a su sabor, bajo contenido calórico y propiedades antioxidantes (2). Para luchar contra las enfermedades de las plantas, se ha aplicado una gran variedad de sustancias; siendo el uso ancestral de extractos vegetales reemplazado por la utilización de sustancias químicas en grandes extensiones de cultivos.
Para el presente trabajo se han escogido tres genes WRKY, que codifican como factores de transcripción y que presentan mayores niveles de expresión en las hojas de tomate (3).

Fotografía 1: Cultivo de plantas de tomate en condiciones controladas de laboratorio.

Objetivos

Determinar la activación de genes de defensa SlWRKY 8, SlWRKY 23 y SlWRKY 39, una vez transcurridas 24 y 48 horas en plantas de tomate de mesa (*Lycopersicum esculentum L.*) mediante la aplicación de 3 tratamientos: una sustancia de origen vegetal como extracto (T 1), de una sustancia química electrolítica (T 2) y de un fungicida de venta comercial (T 3). Comparar y discriminar la eficacia de los tratamientos.

Metodología

Las plantas de tomate de mesa fueron cultivadas bajo condiciones controladas en laboratorio. Una vez aplicados los tratamientos, se procedió a la extracción de ARN, previa congelación del material vegetal en nitrógeno líquido. Se utilizó PCR en tiempo real y cuantificación relativa para el análisis de la expresión de los genes citados confrontando los niveles de expresión con los controles, plantas tratadas con agua (4) (5). Con los resultados obtenidos, se realizó el análisis estadístico ANOVA y la prueba HSD de Tukey para discriminar la efectividad de los tratamientos.

Resultados

Los valores de activación para los genes SlWRKY no son representativos, únicamente SlWRKY 23 se activa alcanzando un valor Fold Change de 4 a 24 horas con el tratamiento de agroquímico. Estos resultados informan la expresión de genes de defensa inducidos por sustancias con acción biocida y la posibilidad de caracterizar otras sustancias que puedan producir esta activación sobre plantas de tomate.

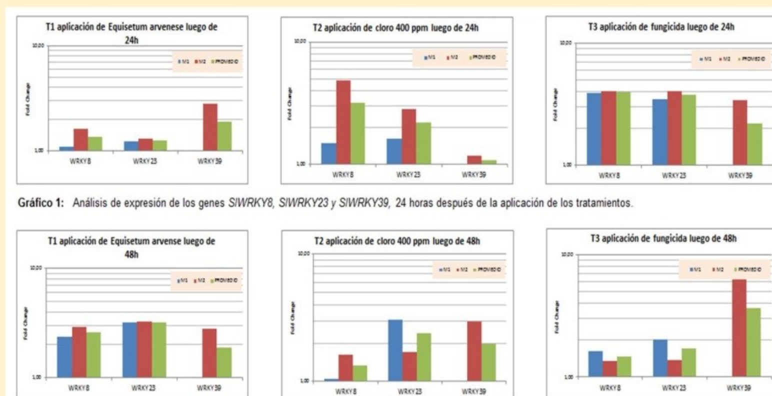
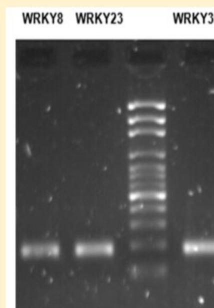


Gráfico 1: Análisis de expresión de los genes *SlWRKY8, SlWRKY23 y SlWRKY39*, 24 horas después de la aplicación de los tratamientos.

Gráfico 2: Análisis de expresión de los genes *SlWRKY8, SlWRKY23 y SlWRKY39*, 48 horas después de la aplicación de los tratamientos.



Fotografía 2: Comprobación en gel de agarosa al 1,5%, de los fragmentos amplificados por qPCR de los genes *SlWRKY8, SlWRKY23, SlWRKY 39*, se encuentran dentro del tamaño esperado, 200 pb, al compararlos con el marcador de peso molecular de 100 pb.

Conclusiones

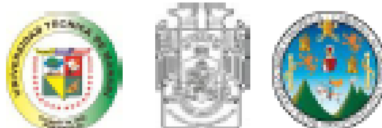
Los genes WRKY codifican factores transcripcionales que intervienen en una gran variedad de procesos en las plantas, entre ellos la respuesta al estrés abiótico. Dependiendo del tratamiento se puede observar una respuesta de activación génica diferente. Para el caso del tratamiento 1 con Equisetum arvense se observa una inducción para todos los genes analizados a las 48 horas, mientras que a las 24 horas no se observa una activación significativa. El tratamiento 2 con cloro, por otro lado, es más efectivo para el gen SlWRKY8 a las 24 horas, mientras que a las 48 horas la activación no es significativa. El fungicida induce una activación génica luego de 24 horas, siendo SlWRKY39 el más activado a las 48 horas. Se muestran diferentes perfiles de expresión entre los tres genes, de cualquier manera los tratamientos con sustancias naturales producen activación de factores transcripcionales relacionados con el sistema de defensa en las plantas.

Bibliografía

1. Guía del Cultivo de Tomate. Juana Pérez, Guillermo Hurtado. San Salvador: s.n.
2. Ministerio de Agricultura, Ganadería, Acuacultura y Pesca. http://sinagap.agricultura.gob.ec/index.php/site-map/2-produccion. [En línea] 2013.
3. Genome-wide analysis of WRKY transcription factor in Solanum lycopersicum, S. Huang et al., Mol. Genet. Genomics 2012, 287:495-513.
4. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Vandesompele et al. Genome Biology, 2002 Jun 18;3(7):RESEARCH0034
5. Cálculo en biología molecular y biotecnología: guía de matemáticas para el laboratorio, Frank H. Stephenson, Jorge Lloberas Cavero, Segunda Edición, Elsevier, Barcelona.

ANNEX E

Letter of acceptance for the lecture at the 4to Simposio Internacional de Investigación Multidisciplinaria, octubre 2014.



UNIVERSIDAD TÉCNICA DE MANABÍ, ECUADOR
UNIVERSIDAD JUÁREZ AUTÓNOMA DE TABASCO, MÉXICO
UNIVERSIDAD DE SAN CARLOS DE GUATEMALA

CARTA DE NOTIFICACIÓN

Estimados Autores:

DRA. INÉS MALO CEVALLOS

El **Comité de Arbitraje** de los trabajos recibidos en respuesta a la convocatoria del 4to. Simposium Internacional de Investigación Multidisciplinaria y 1er. Congreso Internacional: La Investigación al servicio del Buen Vivir, le informan que el trabajo titulado:

ACTIVACIÓN DE GENES DE DEFENSA EN PLANTAS DE TOMATE DE MESA *Lycopersicum esculentum L.*, A TRAVÉS DE LA APLICACIÓN DE SUSTANCIAS QUÍMICAS Y NATURALES"

Ha sido **ACEPTADO** para la sustentación en el marco de este importante evento científico, que se llevará a cabo los días 15, 16 y 17 de octubre de del año en curso en la Universidad Técnica en Portoviejo Manabí, Ecuador. En días posteriores se le hará llegar el programa general en el correo electrónico, en donde podrán verificar la modalidad de presentación (oral o cartel), sala, horario y fecha. Además el programa estará disponible en www.utm.edu.ec y www.ujat.mx. Los trabajos serán incluidos en libro electrónico, con ISBN.

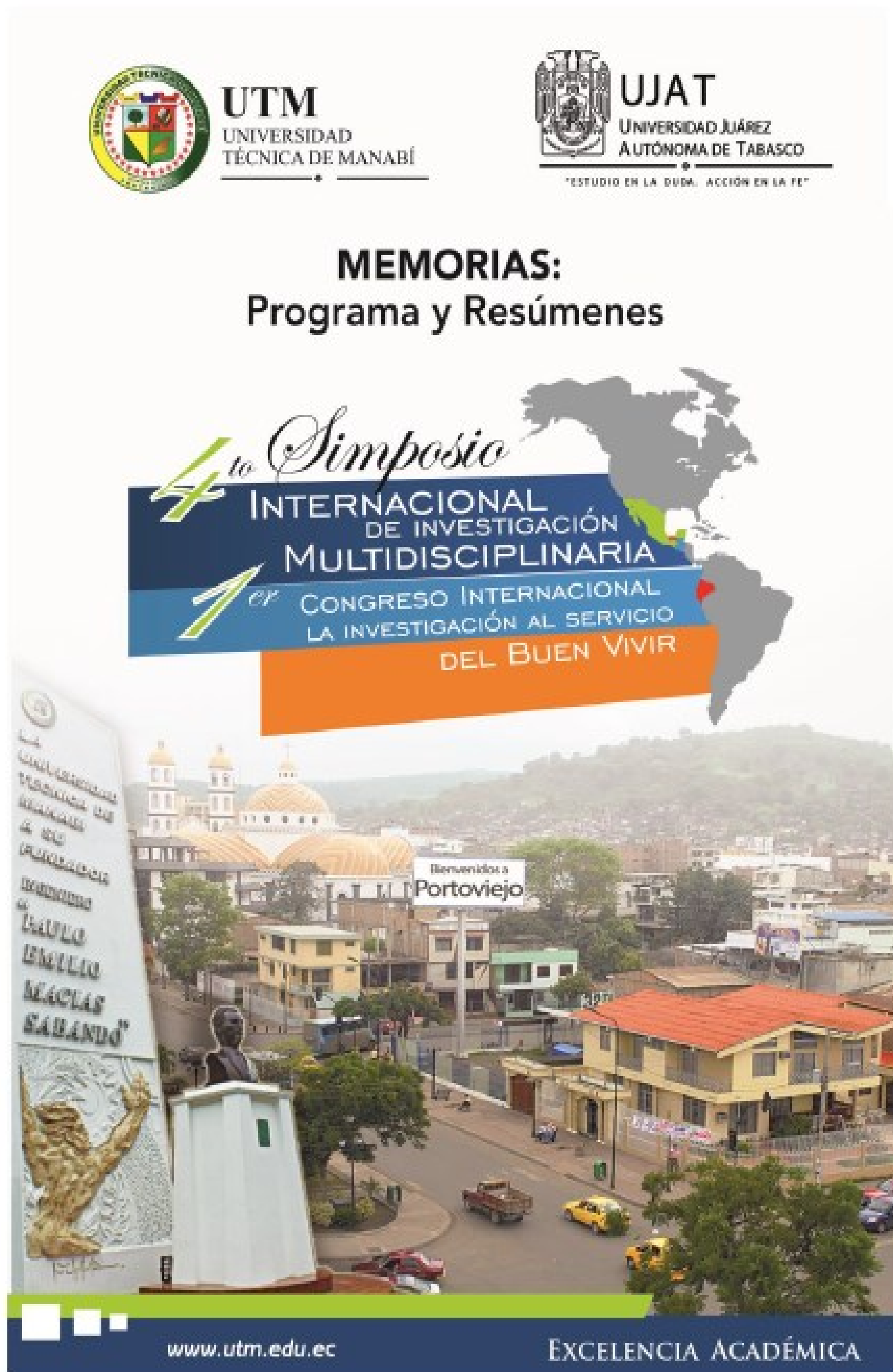
Portoviejo, 26 de Septiembre de 2014

Comité Científico México-Ecuador

UTM (Ecuador) Dirección: Av. Urbina y Che Guevara - Portoviejo - Manabí - Ecuador; Teléfonos: (593-05) 2632677 – 2632692.
UJAT - DAMR (México): Carretera Tenosique – Estapilla km 1 Tenosique, Tabasco, México C.P. 86901, Tel 01(934) 3422110
USAC –CUDEP (Guatemala): Parque Las Estelas, Santa Elena a 1 km del aeropuerto. Forma de acceso por la 4a calle de Santa Elena. Santa Elena, Petén, Guatemala.

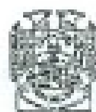
ANNEX F

Cover of the 4to Simposio Internacional de Investigación Multidisciplinaria, october 2014.



ANNEX G

Schedule for the lecture held on Thursday 16, October 2014.



4to. SIMPOSIUM INTERNACIONAL DE INVESTIGACION MULTIDISCIPLINARIA
1er. Congreso Internacional la Investigación al Servicio del Buen Vivir
15-17 Octubre de 2014

JUEVES 16 DE OCTUBRE

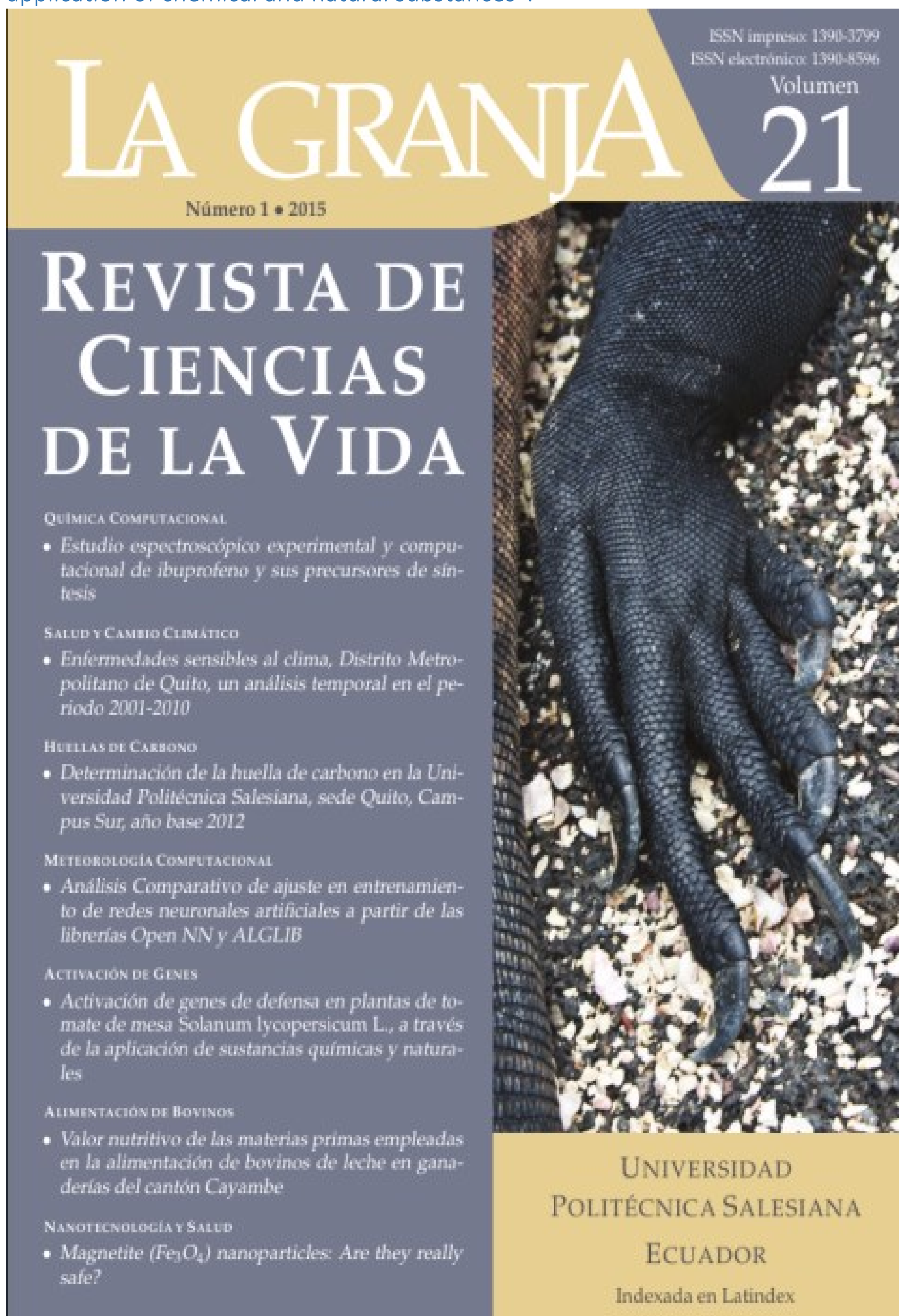
Área: Biotecnología y Ciencias Agropecuarias
LUGAR: AUDITORIO "ING. TITO GOROZABEL TORRES" 1

Clave	Título de la Ponencia	Horario
BCA-07	**Huertos familiares: Una estrategia sustentable para el buen vivir.** Nicolás González Cortés, Emilio Jesús Maldonado Enríquez, Román Jiménez Vera y Martha Isela Baños Dorantes.	11:00 - 11:20
BCA-08	**Diseño y evaluación de Primers para estudio de la expresión del GEN PEPT1 POR RT-PCR en juveniles de tilapia.** Juan Vera Delgado	11:20 - 11:40
BCA-09	**Caracterización del consumo de Pejelagarto (Atractosteus tropicus) y evaluación sensorial de organismos con dos tipos de alimentación.** Gabriela Narváez Ortiz, Carlos Alfonso Álvarez González, Rosa Ma. Salinas Hernández, Hildegard Mayanín Ávalos González.	11:40 - 12:00
	SESIÓN DE PREGUNTAS	12:00 – 12:20
	COMIDA LIBRE	12:20 – 14:00
BCA-10	**Ictioplancton y peces del estuario del Río Chone.** María Laura García Veintimilla, Roberto Retamales González	14:00 - 14:20
BCA-11	**Efecto de un extracto de papaína sobre las características sensoriales y fisicoquímicas de carne ovina y bovina.** María Gabriela Guillén de la Cruz; Rosa Ma. Salinas Hernández, Dora Centurión Hidalgo, Emilio Jesús Maldonado Enríquez	14:20 - 14:40
	SESIÓN DE PREGUNTAS	14:40 – 15:00
	RECESO/COFFEE BREAK	15:00 – 15:20
BCA-12	**Metales pesados y minerales en harina del músculo de Plecostomus (Pterygoplichthys pardalis).** Emilio Jesús Maldonado Enríquez; Ulises López Noverola; Nicolás González Cortés, Rosa María Salinas Hernández	15:20 - 15:40
BCA-13	**Determinación de antioxidantes en mieles tropicales.** Martha María López-González, Enrique Sauri-Duch, Ángel Sol-Sánchez y Juan Manuel Zaldívar-Cruz.	15:40 - 16:00
BCA-14	**"ACTIVACIÓN DE GENES DE DEFENSA EN PLANTAS DE TOMATE DE MESA Lycopersicum esculentum L., A TRAVÉS DE LA APLICACIÓN DE SUSTANCIAS QUÍMICAS Y NATURALES".** Inés Malo Cevallos	16:00 – 16:20
BCA-15	ABSORCIÓN DE CO2 EN EL CULTIVO DE RICINUS COMMUNIS L. (HIGUERILLA) EN LA PROVINCIA DE MANABÍ, ECUADOR. Manuel Ricardo Saltos Giler	16:20 – 16:40
	SESIÓN DE PREGUNTAS	16:40- 17:00

8

ANNEX H

Cover of "La Granja" Volume 21 Magazine, in which is the article: "Activation of Defense Genes in tomato plants, *Lycopersicum esculentum* L., through the application of chemical and natural substances".



ISSN impreso: 1390-3799
ISSN electrónico: 1390-8596

LA GRANJA

Volumen 21

Número 1 • 2015

REVISTA DE CIENCIAS DE LA VIDA

QUÍMICA COMPUTACIONAL
- *Estudio espectroscópico experimental y computacional de ibuprofeno y sus precursores de síntesis*

SALUD Y CAMBIO CLIMÁTICO
- *Enfermedades sensibles al clima, Distrito Metropolitano de Quito, un análisis temporal en el periodo 2001-2010*

HUELLAS DE CARBONO
- *Determinación de la huella de carbono en la Universidad Politécnica Salesiana, sede Quito, Campus Sur, año base 2012*

METEOROLOGÍA COMPUTACIONAL
- *Análisis Comparativo de ajuste en entrenamiento de redes neuronales artificiales a partir de las librerías Open NN y ALGLIB*

ACTIVACIÓN DE GENES
- *Activación de genes de defensa en plantas de tomate de mesa* Solanum lycopersicum *L., a través de la aplicación de sustancias químicas y naturales*

ALIMENTACIÓN DE BOVINOS
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UNIVERSIDAD POLITÉCNICA SALESIANA ECUADOR

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ANNEX I

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Artículo científico / Scientific paper

ACTIVACIÓN DE GENES

LA GRANJA: REVISTA DE CIENCIAS DE LA VIDA

DOI: 10.17163/lgr.n21.2015.05

ACTIVACIÓN DE GENES DE DEFENSA EN PLANTAS DE TOMATE DE MESA *Lycopersicum Esculentum L.*, A TRAVÉS DE LA APLICACIÓN DE SUSTANCIAS QUÍMICAS Y NATURALES

ACTIVATION OF DEFENSE GENES IN TOMATO PLANTS, *Lycopersicum Esculentum L.*, THROUGH THE APPLICATION OF CHEMICAL AND NATURAL SUBSTANCES

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Resumen

Lycopersicum esculentum L. es una planta cuyo cultivo en el país, tanto a cielo abierto como en invernadero, está sujeto a la utilización de agroquímicos para que sea económicamente rentable, puesto que se ve afectado por una gran variedad de plagas y condiciones ambientales adversas. El consumo de tomate de mesa se encuentra muy difundido en nuestro país debido a su sabor, bajo contenido calórico y propiedades antioxidantes. Para luchar contra las enfermedades de las plantas, se han aplicado una gran variedad de sustancias; cabe señalar que el uso ancestral de extractos vegetales ha sido reemplazado por la utilización de sustancias químicas en grandes extensiones de cultivos.

Para el presente trabajo, se escogieron tres genes WRKY, que codifican como factores de transcripción, y que presentan mayores niveles de expresión en las hojas de tomate. Se evaluó la activación de los genes SlWRKY 8, SlWRKY 23 y SlWRKY 39, una vez transcurridas 24 y 48 horas de la aplicación tanto de una sustancia de origen vegetal como extracto (T 1), de una sustancia química (T 2) y de un fungicida de venta comercial (T 3). Las plantas de tomate de mesa fueron cultivadas bajo condiciones controladas. Una vez aplicados los tratamientos, se procedió a la extracción del ARN, se utilizó PCR en tiempo real y cuantificación relativa para el análisis de la expresión de los genes citados. Con los resultados obtenidos, se realizó el análisis estadístico ANOVA y la prueba HSD de Tukey para discriminar la efectividad de los tratamientos.

De los genes WRKY analizados, únicamente SlWRKY 23 se activa alcanzando un valor Fold Change de 4 a las 24 horas con el tratamiento de agroquímico. Estos resultados informan la expresión de genes de defensa inducidos por sustancias con acción biocida y la posibilidad de caracterizar otras sustancias que puedan producir esta activación sobre plantas de tomate.

Palabras claves: tomate, WRKY, factores de transcripción, RT-PCR.

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Abstract

Lycopersicum esculentum L. is a plant whose cultivation in the country—both open and greenhouse cultivation—is subject to the use of agrochemicals to make it economically viable, since it is affected by a great variety of pests and adverse environmental conditions. Tomato consumption is widespread in our country because of its taste, for being low in calories, and its antioxidant properties. A diversity of substances has been used to control plant illnesses, it is worth mentioning that ancestrally the use of vegetable extracts has been replaced by the use of chemicals in large extensions of crops.

For this work, three WRKY genes were chosen, which act as transcription factors, and have higher expression levels in tomato leaves. Gene activation of SlWRKY 8, SlWRKY 23 y SlWRKY 39 was assessed 24 and 48 hours after the application of: substances of plant origin (T 1), electrolytic chemical (T 2) and a commercial fungicide (T 3). The tomato plants were grown under controlled laboratory conditions. Once the treatments were applied, RNA was extracted, real time PCR was performed and also, relative quantification was used to analyze the expression of the aforementioned genes. These results were analyzed statistically using ANOVA and Tukey's HSD test to discriminate the effectiveness of each treatment.

From the analyzed WRKY genes, only SlWRKY 23 showed and activation reaching a Fold Change value of 4 at 24 hours with the agrochemical treatment. These results provide evidence for the expression of induced defensive genes by substances of biocide activity and the possibility to characterize other substances that may cause this activation in tomato plants.

Keywords: tomato, WRKY, transcription factors, RT-PCR

Forma sugerida de citar: Malo, I., G. Bernacchia y P. Arévalo. 2015. **Activación de genes de defensa en plantas de tomate de mesa *Lycopersicum Esculentum L.*, a través de la aplicación de sustancias químicas y naturales.** La Granja: Revista de Ciencias de la Vida. Vol. 21(1): 61-68. ISSN: 1390-3799.

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ANNEX J

Cover of "La Granja" Volume 23 Magazine, in which is the article: "Energy Use Getting Plastic Waste Liquid Fuels By Pyrolysis".

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LA GRANJA

Volumen 23

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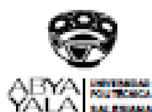
ANNEX K

file:///E:/960-3698-1-PB%20Art%C3%ADculo%20La%20Granja%20Revista%2023.pdf



Artículo científico / Scientific paper

PIRÓLISIS

LA GRANJA: REVISTA DE CIENCIAS DE LA VIDA

DOI:10.17163/lgr.n23.2016.06

APROVECHAMIENTO ENERGÉTICO DE RESIDUOS PLÁSTICOS OBTENIENDO COMBUSTIBLES LÍQUIDOS, POR MEDIO DE PIRÓLISIS

ENERGY USE GETTING PLASTIC WASTE LIQUID FUELS BYPYROLYSIS

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Resumen

Este estudio da a conocer una forma eficiente de gestionar los residuos plásticos obteniendo combustibles a través del proceso de pirólisis de poetilentereftalato, polietileno de alta densidad y poliestireno, residuos plásticos de mayor generación dentro de la Universidad Politécnica Salesiana; en la investigación se determinó que el residuo que mayor porcentaje de fracción líquida produce es el poliestireno. sin embargo, de acuerdo a los análisis el de mejor calidad es el polietileno de alta densidad considerado como crudo mediano. De acuerdo a los análisis de cromatografía, se muestra que las fracciones líquidas del proceso de pirólisis de residuos plásticos contienen sustancias que forman parte de los combustibles y petróleos, lo que confirma la obtención de combustibles líquidos de características semejantes a los tradicionales y que se pueden usar para generar energía en motores de combustión.

Palabras claves: pirólisis, residuos plásticos, combustibles líquidos, poliestireno, polietileno.

Abstract

This study, found an efficient way to manage plastic wastes obtaining fuels; by pyrolysis process at poetilentereftalato, high density polyethylene, polystyrene and high generation plastic wastes at the Universidad PolitÃ©cnica Salesiana; in the investigation was determined that the highest percentage of liquid fraction residue was achieved by polystyrene. however, according to analyzes the best quality was the high density polyethylene considered medium crude. According to chromatographic analysis, this research demonstrates that the liquid fractions of pyrolysis of plastic wastes contain substances that are part of fuels and oils, which confirms the existance of liquid fuels with similar characteristics to traditional ones and can be used in combustion engines.

Keywords: pyrolysis, plastic waste, liquid fuels, polystyrene, polyethylene.

Forma sugerida de citar: Mancheno, M., *et. al.* 2016. **Aprovechamiento energético de residuos plásticos obteniendo combustibles líquidos, por medio de pirólisis.** La Granja: Revista de Ciencias de la Vida. Vol. 23(1): 53–59. ISSN impreso: 1390-3799. ISSN electrónico: 1390-8596.

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ANNEX L

Letter of acceptance for the article "Natural extracts from pepper, wild rue and clove can activate defenses against pathogens in tomato plants", in the European Journal of Plant Pathology, January 2017.

Giovanni Bernacchia<bhg@unife.it>

Responder a todos|

lun 23/01, 11:06

Ines Patricia Malo Cevallos

Inbox

---------- Forwarded message ----------
From: "Jesson Austria (EJPP)" <em@editorialmanager.com>
Date: 23 Jan 2017 10:24
Subject: Your Submission EJPP-D-16-00505R2
To: "Giovanni Bernacchia" <bhg@unife.it>
Cc:

Dear Dr Bernacchia,

We are pleased to inform you that your manuscript, "Natural extracts from pepper, wild rue and clove can activate defenses against pathogens in tomato plants", has been accepted for publication in the European Journal of Plant Pathology.

You will receive an e-mail from Springer in due course with regards to the following items:

1. Offprints
2. Colour figures
3. Open Choice
4. Transfer of Copyright

Please remember to quote the manuscript number, EJPP-D-16-00505R2, whenever inquiring about your manuscript.

With best regards,

M.J. Jeger
Editor in Chief

ANNEX M

First page of the article published in the European Journal of Plant Pathology.

Eur J Plant Pathol
DOI 10.1007/s10658-017-1168-y

Natural extracts from pepper, wild rue and clove can activate defenses against pathogens in tomato plants

I. Malo · M. De Bastiani · P. Arevalo · G. Bernacchia

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Abstract Tomato is an important species grown in many countries, either in fields or greenhouses. Despite decades of improvement, it is still susceptible to diseases, thus requiring the use of chemical pesticides, especially in greenhouses. Nevertheless it is imperative to reduce the use of environmental-unfriendly phytochemicals and favor less toxic tools to fight pathogens. Plants possess elaborate mechanisms against diseases that can lead to resistance. In the present work, we investigate the induction of plant defenses by means of extracts from plants widespread and easy to find, also known for their antimicrobial properties. Aqueous extracts of pepper 'Rocoto', wild rue and ethanolic extracts of clove powder (whose inhibiting effect was assessed on *Oidium* sp. spores) were tested on tomato plants for their ability to induce expression of different defense genes (PRs and regulatory proteins) after spraying. As revealed by RT-qPCR, all extracts were able to induce mRNA accumulation of different PR and MAPK regulators for several hours upon treatment, with clove and wild rue being the strongest. This effect could also be reproduced in tomato plants after a second treatment, 15 days after the first. The same extracts were tested in tomato and tobacco plants via leaf infiltration, showing necrotic symptoms associated with the hypersensitive response, thus confirming the priming capacity of the extracts. The involvement of salicylic acid (SA) in these responses was verified by HPLC analysis and in SA-depleted transgenic tobacco (NahG). The results obtained suggest that natural antimicrobial extracts can be used to induce plant defenses and protect valuable crops. At the same time these low-cost extracts do not pose a threat to the environment or the farmer and can help reduce the farming costs, especially in developing countries.

Keywords Quantitative PCR · Resistance · Antimicrobial · Priming · Pathogenesis-related proteins

Abbreviations

BABA	β-aminobutirric acid
BTH	benzo (1,2,3) thiadiazole-7-carbothioic acid
GUS	β-glucuronidase
INA	2,6-dichloroisonicotinic acid
JA	jasmonic acid
MAPK	mitogen-activated protein kinase
NaDC	sodium deoxycholate
PR	pathogenesis-related proteins
RT-qPCR	reverse transcription quantitative PCR
SA	salicylic acid
WT	wild type

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