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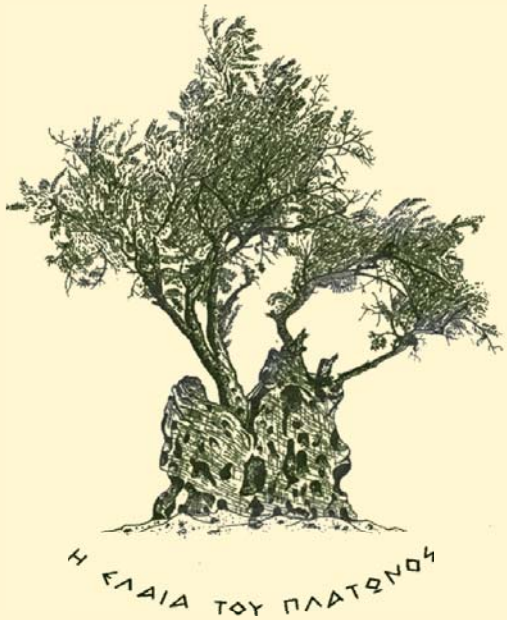

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REVIEW ARTICLE

Molecular advances on agricultural crop improvement to meet current cultivating demands

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Abstract Sunflower, maize and potato are among the world's principal crops. In order to improve various traits, these crops have been genetically engineered to a great extent. Even though molecular markers for simple traits such as, fertility, herbicide tolerance or specific pathogen resistance have been successfully used in marker-assisted breeding programs for years, agronomical important complex quantitative traits like yield, biotic and abiotic stress resistance and seed quality content are challenging and require whole genome approaches. Collections of genetic resources for these crops are conserved worldwide and represent valuable resources to study complex traits. Nowadays technological advances and the availability of genome sequence have made novel approaches on the whole genome level possible. Molecular breeding, including both transgenic approach and marker-assisted breeding have facilitated the production of large amounts of markers for high density maps and allowed genome-wide association studies and genomic selection in sunflower, maize and potato. Marker-assisted selection related to hybrid performance has shown that genomic selection is a successful approach to address complex quantitative traits and to facilitate speeding up breeding programs in these crops in the future.

Additional keywords: Crop improvement, agricultural biotechnology, marker assisted selection, improved agronomic traits

Introduction

Agriculture is a human invention since more than 10,000 years and is estimated to have used more than 7,000 species to satisfy basic human needs (Esquinas-Alcázar, 2005). The primitive crop cultivars, known as landraces, were adapted to local growing conditions and practices, and therefore remained genetically diverse for traits such as product qualities, stress tolerance, disease resistance, and yield stability. Today's agricultural commodities and modern varieties derived from the genetic modification of wild plants through thousands of years of gradual selection, domestication and breeding, are more genetically uniform than their wild relatives (Fu, 2015). Given that plant genetic diversity increases options for innovative, plant-based solutions to major environmental challenges such as water scarcity, deforestation, energy and climate change, molecular plant breeding can be a valuable tool to meet these demands by rapid incorporation of important traits from wild relatives into established crops and by shortening new crop domestication time (da Silva Dias, 2015).

Nowadays affordable high throughput DNA sequencing, coupled with improved bioinformatics and statistical analyses, is bringing major advances in the field of molecular plant breeding. Multidisciplinary breeding programs on the world's major crop plants are able to investigate genome-wide variations in DNA sequences and link them to inherited highly complex traits which are controlled by several genes, such as hybrid vigor and flowering. Furthermore, there has been

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a step-change in speed and cost-effectiveness (Robinson *et al.,* 2014). The availability of dense genetic maps can facilitate researchers to perform flexible marker-trait associations, concerning the correlations between pathogen resistance and alternative genes, and develop high performance markers that will promote marker- assisted choice (MAS) selection for resistant populations in segregating breeding programs (Ben-Ari and Lavi, 2012).

Herein, the molecular advances on agricultural crop improvement to meet current cultivating demands are reviewed for three economically important crops worldwide, i.e. sunflower, maize, potato.

Sunflower (*Helianthus annuus* L., Asteraceae)

Sunflower is the foremost seed crop cultivated within the world (Fernández-Luqueño *et al.,* 2014). Sunflower oil contains less than 11% total saturated fat and does not contain any trans fat. Inexpensive production of biofuel from sunflower oil has been achieved (Boumesbah *et al.,* 2015). Furthermore, sunflower is an ideal plant for producing high quality rubber from its leaves and stems and some of the taller perennial species have high latex yield potential (Lu and Hoeft, 2009).

The multiple usages of sunflower products in food, feed, and industry are stimulating the discovery of new sources of biodiversity for sunflower molecular breeding programs in combination with the application of high throughput approaches and genetic manipulation. The primary objective for sunflower breeders it to increase the yield and agronomical performance of high oleic sunflower hybrids. To accomplish these goals, breeders need to address pathogens, pests, and environmental constraints that have the potential to drastically reduce yield where sunflowers are grown (Dimitrijevic and Horn, 2018).

Genomic resources

A rich and various germplasm assortment is the backbone of each crop improvement program. Assessing genetic diversity within a genetic pool of novel breeding germplasm could make crop improvement more efficient by the directed accumulation of desired alleles (Darvishzadeh *et al.,* 2010). Several bacterial artificial chromosome (BAC) libraries have been constructed for sunflower (Feng *et al.,* 2006; Gentzbittel *et al.,* 2002; Özdemir *et al.,* 2004). The libraries are equivalent to approximately 8 haploid genomes of sunflower and provide a greater than 99% probability of obtaining a clone of interest and they have been employed for isolating and physical mapping of loci such as the FAD2-1 locus (Schuppert *et al.,* 2006) or the fertility restorer Rf1 locus (Hamrit *et al.,* 2008). *In situ* hybridization techniques involving Fluorescent In Situ Hybridization (FISH) and BAC-FISH have being optimized for diversity and biological process studies between species of the genus Helianthus and development of a physical helianthus map allowing a cross reference to the genetic map (Giordani *et al.,* 2014).

Various EST sequencing programs have been carried out in sunflower, including the Compositae Genome Project, and other programs (Tamborindeguy *et al.,* 2004) and (Ben *et al.,* 2005). The Compositae Genome Program (http://compgenomics.ucdavis.edu/index.php) has developed and is utilizing a 2.6 million feature Affymetrix chip based on 87,000 unigenes from seven *Helianthus* spp. (Lai *et al.,* 2012). Interesting associations have been detected between Expressed Sequence Tags (ESTs) and Quantitative Trait Loci (QTLs) for salt tolerance and for domestication traits (Lai *et al.,* 2005). Until today, 94.33 % of HA412-HO ESTs are correctly mapped and 90,935 protein coding genes are predicted, excluding transposable elements (http://www.sunflowergenome.org). Extensive genotyping has been performed for vegetative and flower sunflower organs together with uncovering gene networks for oil metabolism and flowering time (Badouin *et al.,* 2017; Renaut 2017).

Efficient breeding strategy development

Biotechnology has the potential to help

evoke the full potential of this valuable crop (Fig. 1).

Resistance to pathogens

MAS technology has been used in sunflower breeding for various disease resistance traits (Brahm and Friedt 2000). With the development of an array of molecular markers and a dense genetic map of the sunflower genome, MAS for both single genes and QTLs is now possible (Babu *et al.,* 2004; Bowers *et al.,* 2012). For example, biotechnology offers a variety of methods for managing white rot caused by *Stromatinia cepivora* (also known as *Sclerotium cepivorum*) (Schnabl *et al.,* 2002), including defense activation, pathogen inhibition and detoxification (Lu, 2003). According to Hu *et al.* (2003), the enzyme oxalate oxidase can confer resistance against *Sclerotinia sclerotiorum,* (Lib.) de Bary which causes sclerotinia wilt (midstalk rot), in transgenic sunflower plants while according to Sawahel and Hagran (2006), overexpression of a human lysozyme gene in sunflower confers resistance to the pathogen. Recently, the quantitative nature of Sclerotinia resistance has been exploited and QTL analysis showed that different genomic regions may contribute to resistance in different tissues of the plant (Würschum *et al.,* 2014).

Alternative transgenic methods have been developed to reinforce sunflower resistance to diseases. A number of homologues resistance (R) gene have been isolated from sunflower, providing a valuable resource for engineering disease resistance in sunflower (Dimitrijevic and Horn 2018; Hewezi *et al.,* 2006; Qi *et al.,* 2016; Talukder *et al.,* 2016).

Quality traits. Sunflower with high oleic acid content is optimal for the biodiesel industry since the produced oil has up to 90% mono-unsaturated fatty acid concentration, which has high oxidative stability and uniformity. Therefore, producing high concentrations of industrially valuable fatty acids in plant seeds through biotechnological improvements along with modifications of the fatty acid composition can make vegetable oil more versatile for its use (Burton *et al.,* 2004).

One of the challenges for oil composition modification in sunflower is increasing the extent of the new fatty acids. Much work has been performed for the identification of genes involved in primary metabolic pathways and signal transduction at various growth and stress conditions (Liang *et al.,* 2017; Pan *et al.,* 2016; Velasco *et al.,* 2014) to gain insight into the mechanism of antioxidant defense. New genes have been identified and the metabolism of ROS and RNS have been analyzed under various biot-

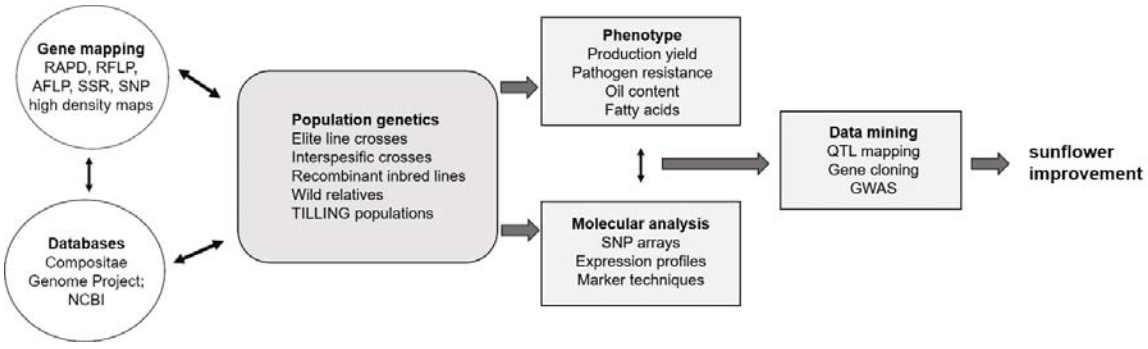

Fig. 1. Schematic depiction of the available resources in sunflower for marker-assisted selection and future genomic selection. Sunflower diverse genetic information is available for breeding and represents a large portion of genetic diversity that can be exploited for improving sunflower traits. Accessing sunflower genome sequences, large resources of SNP or high resolution maps and/or SNP arrays, along with huge amount of expression data can accelerate sunflower breeding by making the selection steps more efficient and precise. Marker-assisted breeding toward genomic selection can produce high quality breeding values.

ic and abiotic conditions (Chaki *et al.,* 2013; Chaki *et al.,* 2008; Chaki *et al.,* 2011).

Overall, transgenic sunflower has the potential to meet the demands for yield improvement, to increase the efficient use of renewable resources, such as land, water and soil nutrients, and to significantly benefit everyday life by providing additional nutritive and healthy foods and valuable industrial products.

Ease of use and robustness of molecular markers

Markers' validation assesses their linkage to and association with QTLs and their effectiveness in selection of the target phenotype in independent populations and different genetic backgrounds (Collard *et al.,* 2005). An overall QTL mapping has been performed using microsatellite and Single Nucleotide Polymorphisms (SNP) markers in sunflower giving the ability to assess the genetic diversity and population structure across different sunflower populations (Filippi *et al.,* 2015).

Validation of genomic Simple Sequence Repeats (SSRs) in four genotypes of sunflower (RHA266, PAC2, HA89 and RHA801) resulted in amplification of 74 sequences from a total of 127 analyzed. Out of them, 13% represented polymorphic loci, 45% monomorphic, 5% null alleles and the remaining 37% showed either no amplification product, nonspecific amplification or complex or difficult to resolve banding patterns (Talia *et al.,* 2010). The percentage of polymorphisms within sunflower that can be genetically mapped using SSR markers is shown to be less than 10% that comes in agreement with reports from other species (Varshney *et al.,* 2005).

Examples of markers/QTLs validation across various genetic backgrounds in sunflower include:

– A set of markers have been validated in a number of different genetic backgrounds for the Or5 gene conferring resistance to race E of the parasitic weed broomrape (*Orobancche cumana*), infecting the sunflower roots (Höniges *et al.,* 2008; Pérez-Vich *et al.,* 2004; Tang and Knapp, 2003).
– Markers have been validated for the dominant PI genes determining resistance to different downy mildew races (Brahm and Friedt 2000; Hvarleva *et al.,* 2009; Ma *et al.,* 2017) and to the R1, Radv and Pu6 genes conferring resistance to rust (Bulos *et al.,* 2014).
– QTLs controlling three resistant (stem lesion, leaf lesion and speed of fungal control) and two morphological (leaf length and leaf length with petiole) traits have been validated for *S. sclerotiorum* across generations (Micic *et al.,* 2005) and across environments (Talukder *et al.,* 2016).
– QTLs have been validated for sunflower oil content, across generations, environments and mapping populations (Tang *et al.,* 2006b).
– Markers have been developed in sunflower for simple traits selection, based on gene mutations underlying the trait of interest. There has been identified a mutation in codon 205 in the acetohydroxyacid synthase gene AHAs-1 that confers resistance to imidazolinone (IMI) herbicides and developed a SNP genotyping assay diagnostic for it (Kolkman *et al.,* 2004).

Maize (*Zea mays* L., Poaceae)

Cultivation of maize is extensively widespread throughout the world and is surpassing any other grains (Council, 2019). With a fraction of total maize production being consumed by humans, its main products are ethanol, animal feed and processed corn starch and corn syrup (Klopfenstein *et al.,* 2013). Maize has high nutritional value but also is a fine source of various major phytochemicals such as carotenoids, phenolic compounds, and phytosterol, depicting its potential health benefits (Rouf Shah *et al.,* 2016).

Genome as the core base

B73 decoding. The 2.3-billion-base genome of an inbred line of maize called B73, an important commercial crop variety has been decoded (Schnable *et al*., 2009). It has

been reported that the Palomero genome, a corn variety diverged from B73 about 9,000 years ago, is around 400 million nucleotides smaller and contains about 20% less repetitive DNA than B732 (Vielle-Calzada *et al.,* 2009). To map maize haplotypes a part of the gene-rich region of 27 maize varieties was sequenced. 'HapMap' revealed thousands of genes around the centres of the chromosomes, where they were unlikely to be shuffled around during recombination (Gore *et al*., 2009). Schnable *et al.* (2011) demonstrated that the maize subgenomes are differentiated by genome dominance and both ancient and ongoing gene loss. Most of the economically important traits considered in maize breeding are inherited quantitatively. Multiple genes or quantitative trait loci (QTLs) affecting flowering traits, root characteristics, cell wall traits, and tolerance to biotic/abiotic stresses panicle morphology and grain development have been cloned, and gene expression research has provided new information about the nature of complex genetic networks involved in the expression of these traits (Buckler *et al*., 2009; Chung *et al*., 2011; Fernandez *et al*., 2009; Messmer *et al*., 2009; Poland *et al*., 2011; Trachsel *et al*., 2009). A meta-analysis of QTL associated with plant digestibility and cell wall composition in maize identified key chromosomal regions involved in silage quality and potentially associated genes for most of these regions (Truntzler *et al*., 2010).

Association mapping (associating specific DNA polymorphisms with traits of interest based on linkage disequilibrium). McMullen *et al.* (2009) described the maize NAM population generated by crossing 25 diverse inbred lines to a common line, inbred B73. Sequenome-based SNP-typing assay was used to identify 1,359 SNPs in maize transcriptome and 75% of these SNPs were confirmed and applied in association analysis (Liu *et al.,* 2010). Currently, there are over 2 million maize ESTs in GenBank (Benson *et al.,* 2009). However, the assembly of these ESTs into gene models presents practical problems. Therefore, a full length cDNA library has been recently constructed for *Zea mays* (http:www.maizecdna.org/) (Soderlund *et al.,* 2009). A normalized cDNA library, covering most of the developmental stages of maize seeds, was also constructed and 57 putative transcription factors were identified (Wang *et al.,* 2010). The cDNA libraries can serve as primary resources for designing microarray probes and as clone resources for genetic engineering to improve crop efficiency.

Maize GDB (http://www.maizegdb.org/). Maize GDB is a database that provides documentation and data for the microarrays produced by the Maize Gene Discovery Project. An extensive expression atlas covering a wide array of tissues and developmental stages of maize using a NimbleGen microarray encompassing 80 301 probe sets was recently constructed (Sekhon *et al.,* 2011). Random-sheared, paired-end Illumina GAII reads have been generated from 103 maize, teosinte and maize landrace inbred lines at a depth ranging from 4-30x (Chia *et al.,* 2012; Hufford *et al.,* 2012). Microarray studies have also been performed to study cell wall metabolism in maize, with the aim of identifying tissue-specific or developmentally regulated gene expression of members of multigene families or to obtain a better understanding of regulatory networks that are exposed when cell wall-related genes are mutated (Guillaumie *et al.,* 2007a; Guillaumie *et al.,* 2007b). The MAIZEWALL sequence database and expression profiling resource has been developed (www.polebio.scsv.ups-tlse.fr/MAIZEWALL). Rajhi and co-workers performed transcriptome analysis in maize root cortical cells during lysigenous aerenchyma formation and discovered a number of genes whose expression changed in response to ethylene under waterlogged conditions (Rajhi *et al.,* 2011).

Maize small RNAs. Small RNAs in the wild type and in the isogenic Mediator Of Paramutation1 loss-of- function (mop1-1) mutant have been examined by deep sequencing to analyze the size distribution of maize small RNAs (Nobuta *et al.,* 2008). Small RNAs are playing roles as major components of epigenetic processes and gene networks

involved in development and homeostasis. It has been recently demonstrated that a change in expression of a key component of the RNA silencing pathway is associated with both vegetative phase change and shifts in epigenetic regulation of a maize transposon (Li *et al.,* 2010).

RNA interference (RNAi) [RNA-mediated gene silencing by sequence-specific degradation of homologous mRNA triggered by double-stranded RNA (dsRNA)]. The RNAi system was used to improve resistance to maize dwarf mosaic virus on transgenic maize (Zhang *et al.,* 2011). Maize lines expressing RNAi to chromatin remodeling factors were shown to be similarly hypersensitive to UV-B radiation but exhibit distinct transcriptome responses (Casati and Walbot 2008). By using near infrared reflectance spectroscopy (NIRS), a set of 39 maize mutants with altered spectral phenotypes ('spectrotypes') have been identified (Vermerris *et al.,* 2007). A number of these mutants were shown to have altered lignin-to-carbohydrate ratios (Penning *et al.,* 2009). Sequence- specific DNA binding Transcription Factors (TFs) are key molecular switches that control or influence many biological processes, such as development or response to environmental changes. The Maize Transcription Factor Database provides a comprehensive collection of 764 predicted transcription factors from maize with available links to information on mutants, map positions or putative functions for these transcription factors (MaizeTFDB) (http://grassius.org/browsefamily.html?species=Maize). Information resources related to metabolomics can play major role not only in metabolomics research but also in synergistic integration with other omics data. MaizeCYc is a biochemical pathway database that provides manually curated or reviewed information about metabolic pathways in maize.

Molecular breeding for current needs

Molecular breeding, including both transgenic approach and marker-assisted breeding, is primary associated with the challenges for developing cultivars with combinations of adaptive traits (Brown *et al.,* 2011; Varshney *et al.,* 2011). For making molecular marker-assisted breeding successful, marker-trait associations are now known for almost all important economic traits, including thousands of mapped microsatellite or SSR markers, and additional recently, SNPs, and insertion-deletion (InDel) markers. For maize, there is an updated compilation of mapped QTL for abiotic stress resistance (http://www.plantstress.com; http://www.maizegdb.org; http://www.gramene.org). Additionally, a large number of genes controlling various aspects of plant development, biotic and abiotic stress resistance, quality characters, etc. have been cloned and characterized in maize, which are excellent assets for molecular marker- assisted breeding (Aslam and Ali 2018; Prasanna *et al.,* 2010).

Tolerance against drought. Since drought is considered to be the most important constraint across all areas where maize is cultivated, and global warming is predicted to further exacerbate drought's impact, a total management plan is necessary for increasing maize yield in stress-prone environments (Fig. 2). The high variability to drought stress and also the uncontrollable fact that drought response has great fluctuations across environments, have made it difficult to spot specific metabolic pathways which limits breeding efforts towards drought tolerance (Collins *et al.,* 2008). A Marker-Assisted BackCross (MABC) selection approach meant for improving grain yield under water limited conditions in tropical maize, was successfully conducted at CIMMYT (Ribaut and Ragot 2006) and more recently at sub-Saharan Africa (Beyene *et al.,* 2016). However, this approach delivers a restricted level of improvement in drought tolerance since it provides an improved version of an existing genotype (Ribaut *et al.,* 2009). Nevertheless, a molecular breeding approach-marker-assisted recurrent selection (MARS) can be used to overcome this problem. MARS studies exploit association mapping and can effectively double the rate of yield gain compared to conventional

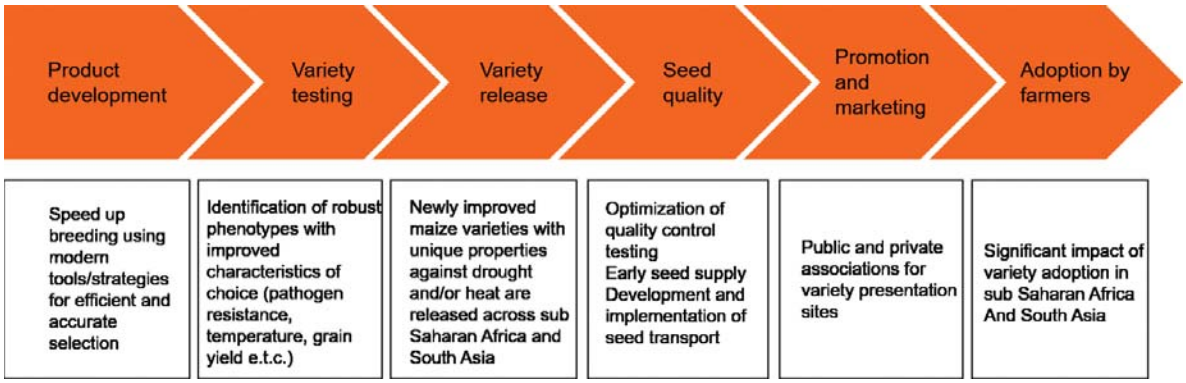

Fig. 2. Schematic representation that highlights the required key steps to facilitate enhanced adoption and impacts of improved climate-resilient maize varieties in the developing world. Increasing maize yields in stress-prone environments and reducing year-to-year variability is an important step in improving food safety, livelihoods and adaptation to the changing climate in the developing world (Cairns and Prasana, 2018).

breeding in elite germplasms when favored and stress environments are been examined (Crosbie *et al.,* 2006; Eathington *et al.,* 2007; Edgerton 2009). Most recently, the role of Abscisic Acid (ABA) pathway in drought resistance has been investigated and natural variants of ABA-(PYR1/PYL/RCAR) protein (PYL) receptors have been identified that can serve as potential molecular markers for breeding drought-resistant maize cultivars (He *et al.,* 2018).

Resistance against pathogens. Efforts to scale down maize losses from pathogen attacks through resistant crop varieties could provide tremendous opportunities for increasing and stabilizing maize productivity. QTL related to resistance to several diseases, such as downy mildew and rust, and insect-pests are known and mapped in maize, creating marker assisted choice as a potentially viable strategy to improve resistance to these biotic stresses (Ali and Yan 2012; García-Lara *et al.,* 2009; Krakowsky *et al.,* 2004; Wisser *et al.,* 2006).

Resistance against insect pests. The industry has made substantial progress with insect resistant maize through transformation with insecticidal proteins from *Bacillus thuringiensis* (Bt) which have been particularly successful in providing protection against several corn borers (Glaser and Matten 2003; Jiang *et al.,* 2018).

Quality traits. Quality traits, like oil content or high nutritional value molecules, have induced a shift in maize production far from strictly an identity-preserved cultivation to more a value-added product. The capability of changing cell membrane polysaccharides into possible sugars for grain ethanol production depends on cell membrane structure. Molecular markers can be a valuable tool when breeding for feed maize but with improved quality on grain ethanol.

QTLs with comparatively efficient results are found for feed maize including cell membrane composition and glucose release (GLCRel) (Lorenzana *et al.,* 2010), and some important constitutive and adaptive QTLs are identified by using meta-analysis (Hao *et al.,* 2010). (Torres *et al.,* 2015) presented the molecular progress that has been made in altering maize's cellulosic content in order to exploit useful biomass characteristics and design new breeding strategies.

Quality traits and tolerance to abiotic stress. There has been increasing interest in addressing advanced traits like grain quality and abiotic/biotic stress tolerances through recombinant DNA technology. Elite inbred South African transgenic corn plants were modified in 3 separate metabolic pathways to produce increased quantities of vitamin β-carotene, ascorbate and folate (Naqvi *et al.,* 2009). It has been demonstrated that engineering of the alkaloid synthesis pathway could have great impact on im-

proving cold tolerance in maize (Quan *et al.,* 2004). Furthermore, genome-wide association analyses (GWAS) in temperate maize inbred lines is serving as a tool to find strategies for identifying genes for cold tolerance (Revilla *et al.,* 2016) and has been reported that the introduction of an antisense gene for pyruvate orthophosphate dikinase (PPDK) into maize with *Agrobacterium*-mediated transformation resulted in shifting the break point 3°C less than that of the wild type (Ohta *et al.,* 2004).

Drought is another stress factor that has been addressed in maize improvement. Nuclear Factor-Y (NF-Y) is a 3- subunit complex that has been shown to play major role in growth, development, and response to environmental stress. Except studies that have been performed for characterizing NF-Y gene families in maize (Zhang *et al.,* 2016), when ZmNF-YB2 or ZmNF-YB16 were constitutively expressed in elite maize inbred lines, the transgenic lines displayed improved drought tolerance compared to wild-type plants under water-stressed conditions in the field (Nelson *et al.,* 2007; Wang *et al.,* 2018). (Castiglioni *et al.,* 2008) demonstrated that transgenic maize lines recombinant with bacterial RNA chaperones resulted in not only abiotic stress tolerance but also improved grain yield under water-limited conditions. The application of this technology has the potential to considerably impact maize production systems that have drought. However, commercialization of transgenic maize for abiotic stresses like drought tolerance has been terribly restricted (Xu *et al.,* 2009).

Moreover, the past ten years we have witnessed extensive efforts toward the development of an efficient *Agrobacterium*-mediated transformation system for an array of maize developing organs with particular emphasis on increasing the efficiency and extending the range of amenable genotypes (Cao *et al.,* 2014; Lee and Zhang 2014; Shrawat and Lörz, 2006).

Validation of quantitative traits

In maize, a trait that has been extensively investigated as an indirect measure of drought tolerance is the capacity of ABA accumulation. The presence of a major QTL for root features (root-ABA1) was mapped on bin 2.04 in Os420 × IABO78. This major QTL affecting abscisic acid (ABA) concentration in the leaf, root traits and relative water content was further evaluated in maize using NILs (Landi *et al.,* 2005). Interestingly, the QTL allele for larger root mass and higher ABA concentration negatively affected grain yield (Landi *et al.,* 2006). Laurie *et al.* (2004) were able to detect 50 QTL accounting for genetic variance in maize oil content with a resolution of the order of a few centimorgans across generations.

QTL conditioning resistance to plant pathogens (rQTL) have been discovered and reviewed by several authors (Balint-Kurti and Johal, 2009; Redinbaugh and Pratt, 2009). To date only a few QTL conferring resistance to maize streak mastrevirus, *Cercospora zeae-maydis*, *Exserohilum turcicum* (Pass.) and *Peronosclerospora sorghiin* have been validated (Abalo *et al.,* 2009; Asea *et al.,* 2009; Nair *et al.,* 2005). For Cercospora resistance in maize, QTLs have been validated across genetic backgrounds (Pozar *et al.,* 2009) and environments (Juliatti *et al.,* 2009). Furthermore, a major QTL controlling maize streak virus resistance explains 50–70% of total phenotypic variation (Pernet *et al.,* 1999). Several microsatellite markers associated with this QTL were validated across populations and have been successfully used for the selection of resistant lines (William *et al.,* 2007).

Analyses for evaluating the significance of QTL x genetic background interactions in several diverse mapping populations, have been performed in maize for grain moisture, silking date and grain yield (Blanc *et al.,* 2006; Huo *et al.,* 2016). QTL meta-analysis is another approach to identify consensus QTL across studies, to validate QTL effects across environments/genetic backgrounds, and also to refine QTL positions on the consensus map (Goffinet and Gerber 2000). The concept of meta-analysis has been applied to the analysis of QTL/genes for flowering

time (Chardon *et al.,* 2004) and drought tolerance in maize (Hao *et al.,* 2010). A meta-analysis of QTL associated with plant digestibility and cell wall composition in maize has been carried out and fifteen meta QTL with confidence interval (CI) smaller than 10cM were identified (Truntzler *et al.,* 2010).

Potato (*Solanum tuberosum,* L., Solanaceae)

Cultivated potato is the world's third most important human food crop (www.cipotato.org). It is also used as raw material for starch and alcohol production (Cantos-Lopes *et al.,* 2018). The basic chromosome number for potato species is 12. Even though one of the most widespread food crop around the world, the genetics of many potato traits is poorly understood.

Insights in genomic properties

An ultrahigh-density (UHD) genetic map composed of approximately 10,000 Amplified Fragment Length Polymorphism (AFLP) markers has been developed, which is most likely the densest map for a plant species ever constructed (Van Os *et al.,* 2006). Recently, the relationship between the genetic and chromosome map in potato was displayed and two linkage maps were integrated with potato genome sequence developing 8303 Single Nucleotide Polymorphism (SNP) for genome-guided breeding (Felcher *et al.,* 2012). Moreover, (Sharma *et al.,* 2013) elaborated 2469 marker loci in a linkage map which was integrated with potato reference genome (DM) and other physical and genetic maps of potato providing detailed information about chromosomal gene distribution. Using RFLP and AFLP markers, a QTL and linkage map of two segregating diploid populations previously evaluated for sugar content after cold storage, was generated. Ten potato genes with unknown function in carbon metabolism or transport were mapped and tested for their effects on sugar content. Results displayed linkage between glucose, fructose and sucrose QTLs and all of eight candidate gene loci (AGPaseS, AGPaseB, SbeI, GapC, Invap, Ppa1, Sut1, Sut2) (Menéndez *et al.,* 2002). Several QTLs affecting the ability to form tubers under long photoperiods (earliness) have been identified (Šimko *et al.,* 1999). A functional map for pathogen resistance, enriched with RGA (resistance gene analog) and DRL (defence related locus) sequences, SNPs and insertion-deletion polymorphisms (InDels) tightly linked or located within Nucleotide Binding Site - Leucine Rich Repeat (NBS-LRR) -like genes, has been developed on the basis of two potato populations (BC9162 and F1840) (Rickert *et al.,* 2003; Trognitz *et al.,* 2002). Recently, twenty-one QTL and eight reference published potato maps were merged together and the first consensus map was built. Individual QTLs for resistance to the late blight pathogen, *Phytophthora infestans* (Mont.) de Bary, and maturity traits were projected onto the consensus map and the first meta-analysis performed deals with both development trait and resistance to a biotic stress in potato (Danan *et al.,* 2011).

As a major follow-up, the genome of potato (850 Mb) was sequenced by the international Potato Genome Sequencing Consortium (PGSC), which was comprised by 13 countries [http://www.potatogenome.net/]. The new genome sequence data provides information about extensive copy number variation (CNV) which has great impact on 219.8 Mb (30.2%) of the potato genome. Almost 30% of genes are subjected to at least partial duplication or deletion which reveals the highly heterogeneous nature of the potato genome (Hardigan *et al.,* 2016). Comparative sequence analysis of *Solanum* and *Arabidopsis* in a hot spot for pathogen resistance on potato chromosome V has also been performed and revealed a patchwork of conserved and rapidly evolving genome segments (Ballvora *et al.,* 2007).

Several efforts to generate EST resources for potato have been performed (Flinn *et al.,* 2005). Potato cDNA microarray analysis was performed to assess the potential of transcriptomics to detect differences in gene expression due to genetic differences or environmental conditions (van Dijk *et al.,* 2009). A

cDNA- AFLP approach and bulked segregate analysis (BSA) was used to identify genes co-segregating with earliness of tuberization in a diploid potato population. 81 candidate polymorphic transcript-derived fragments (TDFs) showing polymorphism between the early and late bulks were selected for further analysis (Fernández-del-Carmen *et al.,* 2007). Genetic engineering could enhance desirable characteristics of crops by modifying key regulatory steps for entire metabolic or developmental pathways. The optimal conditions for genetic transformation of Solanum spp mediated by *Agrobacterium tumefaciens* have been established (Chakravarty *et al.,* 2007). It has been demonstrated that transgenic katahdin plants containing the RB gene showed resistance to all tested *Pythophtora* isolates, including a super race that can overcome all eleven known R genes in potato. An RNA interference (RNAi)-based potato gene silencing approach using agroinfiltration, has been recently established (Bhaskar *et al.,* 2009).

How to design efficient breeding strategies

Tolerance to salt stress. Potato crop production is highly inversely connected to salt stress with substantial economic impacts (Katerji *et al.,* 2000). When potato is subjected to salt stress, increased activation of antioxidant enzymes, accumulation of proline, decrease in micro tubers and negative effects on physiological characteristics occur (Rahnama and Ebrahimzadeh 2004; Tang *et al.,* 2006a; Zhang *et al.,* 2005). Gene expression studies on potato cultivars under different stress conditions, such as cold, heat or salt, revealed that transcription factors, signal transduction factors and heat shock protein (HSP) are associated with abiotic stress responses (Rensink *et al.,* 2005; Tang *et al.,* 2016). In addition, when Δ-pyrroline-5-carboxylase synthetase, which is involved in proline production, is overexpressed, it confers salt tolerance to potato (Hmida-Sayari *et al.,* 2005).

Aghaei *et al.* (2008) examined closely in a protein level the differences between a salt tolerant and a salt sensitive potato culture. They pointed out that among the proteins that were differentially expressed photosynthesis- and protein synthesis-related proteins were drastically down-regulated, whereas osmotine-like proteins, type VI secretion immunity protein (TSI-1), heat-shock proteins, protein inhibitors, calreticulin, and five novel proteins were remarkedly up-regulated. Under salt conditions, major changes occur within the photosytem protein machinery and the Calvin cycle as demonstrated by an in-depth cDNA microarray map constructed from potato leaves (Legay *et al.,* 2009).

More recently, advances have been made in identifying several genes that play key roles to biotic and abiotic stress responses. A pathogen-related protein, named PR-10a, has been identified which is not only induced under biotic stress conditions in potato, but also exhibits significantly increased tolerance under salt and osmosis conditions (El-Banna *et al.,* 2010). Two different studies showed that the metal zinc finger protein St ZFP1 could participate to salt associated potato responses through the ABA- dependent pathway (Tian *et al.,* 2010) and also the cinnamyl alcohol dehydrogenase ibCAD1 may play a very important role in each abiotic and biotic stress resistance mechanisms (Kim *et al.,* 2010).

Tolerance to drought. Another major abiotic stress issue that ends up in crop losses in potato cultivars, is drought. The development of drought tolerant cultivars is of primary importance for maintaining yields beneath temperature change conditions and for the extension of cultivation to sub-optimal cropping areas. Extensive cDNA microarray analysis showed that a tolerant accession to drought, named 397077.16, presented differentially expressed genes when compared to a sensitive variety (Legay *et al.,* 2011). The genes belonged to groups of carbohydrate metabolism, cell protection and detoxification, meaning that the tolerant accession can respond more efficiently to stress and be more adaptive when compared to the sensitive one. Additionally, the work of other groups identified a transcrip-

tion factor which is involved in the activation of drought related genes (Shin *et al.,* 2011) and showed the importance of the overexpression of the L-gulono-c-lactone oxidase (GLOase gene) gene to the resistance to various abiotic stress factors (Upadhyaya *et al.,* 2009).

Resistance to pathogens. The use of resistant varieties is taken into account to be the foremost appropriate approach for the management of *Phytophthora infestans*. Extensive examination of potato genotype SD20 revealed WRKY domain transcription factor (WRKY), single AP2/ERF domain transcription factor (ERF), MAP kinase (MAPK), and NBS-LRR gene families that play essential role in late blight (Yang *et al.,* 2018). Moreover, it has been suggested that the R8 gene, found in field trials, is responsible for late blight resistance and that its mapping on the long arm of chromosome IX along with the generation of markers would be a helpful tool for marker assisted breeding (Jo *et al.,* 2011). Nowadays, R8 gene is a worldwide tool for late blight resistance (Vossen *et al.,* 2016). The introduction of simultaneously three resistance genes from three potato accessions to a sensitive cultivar (Zhu *et al.,* 2012), the silencing of six S-genes in the susceptible potato cultivar Desiree (Sun *et al.,* 2016) or the contribution of R-gene dosage and biochemical pathways to resistance (Gao and Bradeen 2016), are good examples in the literature, considering transformation techniques for late blight resistance. On the other hand, since potato late blight resistance has been thoroughly studied, an extensive map of QTLs and Rpi-genes (resistance genes to *Phytophthora infestans*) has been generated (Danan *et al.,* 2011; Jiang *et al.,* 2018; Stefańczyk *et al.,* 2017).

Other efforts to increase potato resistance to pathogens include exploitation of inhibitor genes. (Khadeeva *et al.,* 2009) showed that transformation of potato plants with an inhibitor gene of buckwheat provides protection to the plants against pathogens. Furthermore, a gene family that function against nematode infections have been sequenced and characterized from *Solamun tuderosum* cv. Desiree (Turra *et al.,* 2009). Also, advances have been made in the identification of genes that are involved in the mechanisms controlling the arbuscular mychorrhizal establishment by the regulation of plant defense genes (Gallou *et al.,* 2012).

Molecular markers as a key tool for crop improvement

Tuber susceptibility to bruising. Diagnostic markers for tuber bruising and enzymatic discoloration, which are very crucial characteristics to crop quality of the cultivated potato, have been validated (Urbany *et al.,* 2011). The markers diagnostic for increased or decreased bruising susceptibility is expected to facilitate the combination of superior alleles in breeding programs.

Potato germplasm (use of sources of resistance to pests and diseases in order to breed varieties cheaper to grow). Although the actual copy number of the genes is not known, DNA markers located close to genes that encode resistance or hypersensitive response to the Potato virus Y (PVY), which can reduce yield up to 80 percent while being relatively symptomless, have been identified and validated (Fulladolsa *et al.,* 2015; Szajko *et al.,* 2014; Tomczyńska *et al.,* 2014). Furthermore, Cleaved Amplified Polymorphic Sequences (CAPs) and Sequence Characterized Amplified Regions (SCARs) have allowed the breeding of genotypes resistant to PVY (Kasai *et al.,* 2000).

The successful employment of four PCR-based diagnostic assays to combine the Ry adg gene for extreme resistance to PVY with Gro1 for nematode resistance and with Rx1 for extreme resistance to potato virus X (PVX, genus Potexvirus), or with Sen1 for wart resistance (*Synchytrium endobioticum*) has been reported (Gebhardt *et al.,* 2006).

The availability of DNA-based markers, which are easy to score, cost-effective and diagnostic for resistance to Pathotypes 2/3 (Pa2/3) of the most significant soilborne pests of potato, the potato cyst nematode (*Globodera pallida*), would greatly speed up the process of new variety development. A

set of markers have been validated for QTL on linkage group IV (renamed GpaIV adg s) across a wide range of germplasm (Moloney *et al.,* 2010).

Field resistance to *Phytophthora infestans* has been characterized in a potato segregating family of 230 full-sub progenies derived from a cross between two hybrid *S. phureja x S. stenotomum* clones. QTLs have been identified and validated for the new genetic loci in this diploid potato family contributing to general resistance against late blight (Costanzo *et al.,* 2005).

Potato breeding widely exploits molecular techniques for generation and conservation of advanced clones, increasing the potato cultivar number every year (Fig. 3). Reliable maintenance of large culture collections is becoming more problematic and a rapid and robust method for variety differentiation is becoming highly desirable. The validation of a set of six SSRs markers that can be used to differentiate over 400 potato cultivars has been reported (Reid and Kerr, 2007).

Prospects

Genomic research allows high-throughput analysis for crop improvement. Genetic markers designed to cover a genome extensively allow not only identification of individual genes associated with complex traits by quantitative trait loci analysis but also the exploration of genetic diversity with regard to natural variations.

Wild relatives are valuable knowledge that can upscale with valuable traits the crop species. Nowadays, only a little fraction is exploited for crop improvement. One of the basic issues of crop improvement is to access the genetic variation from such wild species. This is particularly important to the transfer of valuable, novel genes from wild

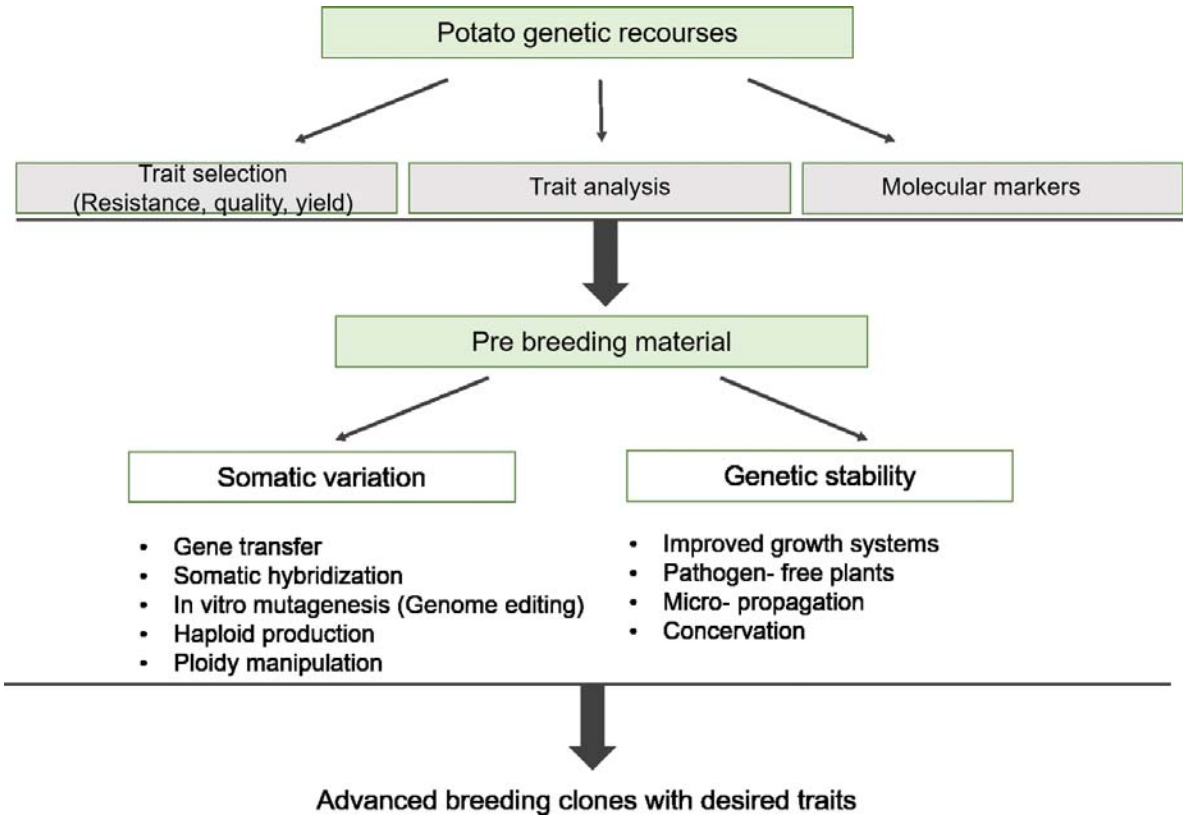

Fig. 3. Gene variants are a valuable tool for improving potato cultivars. Schematic overview of the individual sections that constitute the integrated management of potato genomic resources for the generation of elite breeding clones with improved agronomical traits of interest.

relatives to crops for non-food uses. Biotechnology offers the greatest potential in contributing solutions to problems that agriculture is facing now and the years to come.

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ΑΡΘΡΟ ΑΝΑΣΚΟΠΗΣΗΣ

Μοριακές πρόοδοι στη βελτίωση των γεωργικών καλλιεργειών για την κάλυψη των σύγχρονων απαιτήσεων στη γεωργία

Θ. Μαργαριτοπούλου και Δ. Μηλιώνη

Περίληψη Ο ηλίανθος, ο αραβόσιτος και η πατάτα, είναι μεταξύ των σημαντικότερων καλλιεργειών στον κόσμο. Προκειμένου να βελτιωθούν διάφορα χαρακτηριστικά τους, οι καλλιέργειες έχουν υποστεί γενετική τροποποίηση σε μεγάλο βαθμό. Αν και οι μοριακοί δείκτες έχουν χρησιμοποιηθεί με επιτυχία για την ταυτοποίηση απλών χαρακτηριστικών, όπως η γονιμότητα, η ανοχή σε ζιζανιοκτόνα ή η αντίσταση στα παθογόνα, σημαντικά αγρονομικά χαρακτηριστικά, τα οποία είναι πολύπλοκα και πο-

σοτικά, όπως η απόδοση, η αντοχή σε συνθήκες στρες από βιοτικούς και αβιοτικούς παράγοντες και η ποιότητα του σπόρου, παραμένουν μία πρόκληση και απαιτούν προσεγγίσεις που περιλαμβάνουν τη μελέτη ολόκληρου του γονιδιώματος. Γενετικό υλικό για αυτές τις καλλιέργειες διατηρείται σε τράπεζες σε παγκόσμια κλίμακα και αντιπροσωπεύει πολύτιμους πόρους για τη μελέτη σύνθετων χαρακτηριστικών. Σήμερα, οι τεχνολογικές εξελίξεις και η δυνατότητα αλληλούχησης ολόκληρων γονιδιωμάτων έχουν καταστήσει εφικτές νέες προσεγγίσεις στο επίπεδο του γενώματος. Η μοριακή βελτίωση, συμπεριλαμβανομένων τόσο των διαγονιδιακών μεθόδων όσο και της βελτίωσης με τη βοήθεια γενετικών δεικτών, διευκόλυνε την ταυτοποίηση δεικτών για γενετικούς χάρτες υψηλής πυκνότητας και επέτρεψε μελέτες συσχέτισης ολόκληρου του γονιδιώματος και τη γονιδιακή επιλογή στον ηλίανθο, τον αραβόσιτο και την πατάτα. Η επιλογή μέσω γενετικών δεικτών σχετιζόμενων με τις αποδόσεις υβριδίων έχει δείξει ότι η γονιδιωματική επιλογή είναι μια επιτυχημένη προσέγγιση για την αντιμετώπιση σύνθετων ποσοτικών χαρακτηριστικών και μπορεί να διευκολύνει την επιτάχυνση των προγραμμάτων αναπαραγωγής σε αυτές τις καλλιέργειες στο μέλλον.

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REVIEW ARTICLE

Multistrain versus single-strain plant growth promoting microbial inoculants - The compatibility issue

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Summary Plant Growth Promoting Microorganisms or Plant Probiotics (PGPMs) constitute a promising solution for agricultural sustainability. The concept that inoculation of PGPM mixtures may perform better in enhancing agricultural production than single strain application dates back to the discovery of plant growth rhizobacteria (PGPR) and is gaining ground in our days. This shift is highlighted by the increasing number of research publications dealing with the positive impact of microbial mixtures in promoting plant growth, controlling plant pathogens, as well as providing abiotic stress tolerance. The continuous deposition of patents as well as commercially available formulations concerning bioprotective and/or biostimulant multistrain mixtures also underlines this shift. A major issue in engineering an effective and consistent synthetic multistrain mixture appears to be the compatibility of its components. The present review provides a thorough literature survey supporting the view that treatment of plants with compatible multistrain mixtures generally exerts a better effect in plant growth and health than single-strain inoculation. Our study focuses on multistrain mixtures based on *Pseudomonas*, *Bacillus* and beneficial fungal strains, while commercial products are also being referred.

Additional keywords: plant probiotics, biostimulants, synthetic multistrain mixtures, biological control, co-inoculation, consortia

Introduction

The plant microbiome is composed of active microorganisms that can alter plant physiology and development, perform biological control against pathogens as well as provide tolerance to various types of stress such as drought, salinity, or contaminated soils (Müller *et al*., 2016). These plant associated microbes can be rhizospheric, epiphytic or endophytic with overlap existing between these categories (Turner *et al*., 2013). However, such functions are not carried out by 'the whole microbiome', but by one or a few microbial species acting individually or in a cooperative manner (Hassani *et al*., 2018). These microbes are defined as Plant Growth Promoting Microorganisms (PGPMs) or Plant Probiotics (PPs) (Berg, 2009; Berlec, 2012; Abhilash *et al*., 2016). Plant growth promotion can be direct through production of phytohormones or facilitation of nutrient bioavailability and indirect through biological control of plant pathogens by biological control agents (BCAs). Therefore, the purposeful introduction of PGPM inoculants to plants' microbiome represents an environmentally sound option that holds a prominent position for several decades, in an effort to reduce the overuse of chemical pesticides and fertilizers (Adesemoye and Kloepper, 2009; Abhilash *et al*., 2016; Aloo *et al*., 2019).

In most cases, effective microbial inoculants consist of a single strain. However, the current research trend is shifted towards the development of synthetic bacterial and/or fungal multistrain mixtures with the rationale that they would perform better than single strains (Vorholt *et al*., 2018; Woo and Pepe, 2018). Although single application

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could be effective, mixed inoculants could theoretically adapt to a broader range of environmental conditions and may possess a variety of modes of action (Guetsky *et al*., 2002; García *et al*., 2003; Sarma *et al*., 2015).

In the last two decades, hundreds of studies have been conducted evaluating synthetic mixtures of bacterial species, fungal species or both as plant growth promoting or biological control agents. The concept that combination of beneficial microbial isolates may enhance the efficacy achieved by single isolates dates back to the discovery of Plant Growth Promoting Rhizobacteria (PGPR) (Kloepper *et al*., 1980). In the majority of studies, microbes used to develop microbial mixtures were selected based on their individual PGP activities and/or disease suppressive ability. Then, microbes were mixed together on the assumption that the consortium will be more effective against tested pathogens or in promoting plant growth, without taking into account that antagonistic interactions occurring among PGPMs of the mixture might reduce the expected effects (Sarma *et al*., 2015). Thus, the old issue of compatibility among microbial strains (Kloepper *et al*., 2004) regained a strong position in developing effective multistrain mixtures to use as inoculants (Sarma *et al*., 2015).

Human and animal multistrain probiotics have received more attention than plant probiotics in the past decade. Several multistrain probiotics are being used for human health, animal feed and aquaculture (Markowiak and Śliżewska, 2018; Sniffen *et al*., 2018). However, major issues remain unresolved; whether single strains or multistrain mixtures are considered more beneficial and whether strains in a mixture are compatible with each other (Korada *et al*., 2018; Ouwehand *et al*., 2018). The present study will describe the research findings on the evolution of PGPM mixtures and the compatibility issue among their components in order to provide valuable knowledge for the development of effective microbial mixtures for sustainable agricultural applications.

In vitro compatibility of PGPMs in the construction of multistrain mixtures

Based on a large number of studies, multistrain PGPM mixtures appear to have greater efficacy on improvement of plant growth and/or biological control than single strains. According to the current trend, prerequisites for successful construction of artificial microbial mixtures are: 1) use of diverse microorganisms that can promote plant growth and protect plants from biotic or abiotic stress, 2) efficacy of seed, leaf or root colonization, 3) compatibility among strains in the mixture, 4) use of microorganisms with different modes of action, 5) human and environmental safety, 6) easy application and 7) easy incorporation in an existing management system (Raupach and Kloepper, 1998; Sikora *et al*., 2010; Bashan *et al*., 2014; Großkopf and Soyer, 2014; Ahkami *et al*., 2017)

The issue of compatibility among microbial components of a probiotic multistrain mixture is gaining ground and is considered a basic requirement in the engineering of synthetic microbial mixtures applied to plants (Sarma *et al*., 2015; Friedman *et al*., 2017; Woo and Pepe, 2018) or humans and animals (Ouwahand *et al*., 2018). According to the established literature, the microbial components of a PGPM mixture are considered to be compatible when they have no growth suppressive effect on each other during their *in vitro* co-culture, either in contact or in proximity, or during the plant rhizosphere colonization competition assay (Jain *et al*., 2012; Castanheira *et al*., 2017; Pangesti *et al*., 2017; Santiago *et al*., 2017; Liu *et al*., 2018). In broader terms, compatibility between strains may be achieved when one strain produces toxic compounds and the second strain possesses a detoxifying mechanism that could lead to a certain tolerance of the compounds and vice versa (Kelsic *et al*., 2015; Kamou *et al*., 2016).

In many cases, the outcome of the *in vitro* co-culture compatibility tests reflects the actual nature of the interaction to some extent (Prasad and Subramanian, 2017). For example, competitive colonization assays

under controlled, greenhouse or field conditions demonstrated that *in vitro* compatible bacterial and/or fungal stains are also compatible in the rhizosphere; root population levels reached by each strain in the mixture were not significantly different from those obtained when strains were applied individually (Agusti *et al*., 2011; Alizadeh *et al*., 2013; Stefanic *et al*., 2015; Castanheira *et al*., 2017; Molina-Romero *et al*., 2017; Santiago *et al*., 2017). The same goes with *in vitro* incompatible combinations. For instance, the antagonistic strain of an *in vitro* co-culture may interfere with the root colonization capacity of the other strain (Anith *et al*., 2011; Stefanic *et al*., 2015; Pangesti *et al*., 2017; Santiago *et al*., 2017; Maroniche *et al*., 2018; Varkey *et al*., 2018). Thus, co-inoculation with *in vitro* incompatible strains may result in preventing one or both microbial agents to reaching the appropriate population threshold for plant-beneficial effects (Haas and Defago, 2005).

However, the outcome of the *in vitro* compatibility test does not always represent the actual antagonistic potential in plant conditions (Becker *et al*., 2012). It has been reported that variations in media used to test *in vitro* compatibility may affect the interaction (Georgakopoulos *et al*., 2002; Simoes *et al*., 2008; Deveau *et al*., 2016; Lyons *et al*., 2017). Also, microbes could colonize different ecological niches (Pliego *et al*., 2008), suggesting that *in vitro* incompatible microbes may not interfere with each other's growth on the root surface. In a study of Ruano-Rosa *et al.* (2014) a mixture of *Pseudomonas pseudoalcaligenes* AVO110 and *Trichoderma atroviride* CH 304.1 appears as a very effective combination against *Rosellinia necatrix* to control avocado white root rot, in spite of their observed *in vitro* incompatibility. In another study, the compatible biocontrol agents *Bacillus subtilis* CA32 and *Trichoderma harzianum* RU01 were added together via different modes of application, seed bacterization and fungal soil inoculation, and provided protection from *Rhizoctonia solani* (Abeysinghe, 2009). Abeysinghe (2009) and Ruano-Rosa *et al.* (2014) suggested that mixtures of bacteria and *Trichoderma* strains should be applied at different times and types of inoculation. Also, Anith *et al.* (2011) showed that sequential inoculation of *T. harzianum* and *Piriformospora indica* can increase the coexistence and the beneficial effects on black pepper. In some cases, the biological control agents of a microbial mixture may show *in vitro* compatibility but can be mechanistically incompatible in the sense that one strain interferes with the mechanism by which a second strain suppresses plant disease (Stockwell *et al*., 2011).

Multistrain PGPM mixtures based on *Pseudomonas* or *Bacillus* strains

A major group of PGPMs possessing many traits that make them well suited as biocontrol and plant growth promoting agents is *Pseudomonas* and *Bacillus* bacterial strains. Isolates from both taxa show a wide range of plant beneficial properties such as efficient plant colonization, plant growth promotion, biological control of phytopathogens and induction of plant tolerance to abiotic stress, through mechanisms including production of phytohormones, antibiotic compounds and enhancement of nutrient bioavailability (Hol *et al*., 2013; Aloo *et al*., 2018).

Pseudomonas-based multistrain mixtures

An early study by Sivasithamparam and Parker (1978) showed that co-inoculation of five *Pseudomonas fluorescens* isolates in unsterile soil were highly efficient in reducing the take-all wheat disease caused by *Gaeumannomyces graminis* var. *tritici* while none of the isolates produced a similar effect when tested singly. These data raised the hypothesis that multiple *P. fluorescens* isolates may provide greater and more consistent disease suppression when applied as a mixture than the same strains used individually. This hypothesis was strengthened by the report of Weller and Cook (1983) where

high suppression of this disease was demonstrated after seed treatment with a mixture of *P. fluorescens* strains. Pierson and Weller (1994) using a large number of *P. fluorescens* strains constructed different mixtures, consisting of three or five isolates and demonstrated that only a limited number of mixtures have the potential of greater biocontrol activity against *G. graminis* var. *tritici* compared with the same strains applied individually. However, *in vitro* antagonistic studies of the effective mixtures revealed that their components were either strongly inhibitory to or strongly inhibited by other members of the mixture. A mixture of four or eight *P. fluorescens* genotypes (CHA0, PF5, Q2-87, Q8R196, 1M1-96, MVP1-4, F113 and Phl1C2) producing 2,4-diacetylphoroglucinol (2,4-DAPG) protected tomato plants from *Ralstonia solanacearum* with greater efficacy than single application, although it consisted of strains that *in vitro* inhibited the growth of one or more members of the mixture (Becker *et al*., 2012; Hu *et al*., 2016). However, in other studies, incompatible *P. fluorescens* mixtures of high genotypic richness performed much worse than single strain inoculation (Jousset *et al*., 2014; Mehrabi *et al*., 2016), suggesting that antagonistic activity among the members of the mixture can lead to neutral or negative effect in the inhibition of the pathogen. Hence, the question raised is whether the antagonistic activity of the introduced strains in the rhizosphere enhances the expression of traits involved in disease control or, in contrast, leads to population reduction that consequently diminishes its synergistic effect in controlling the disease.

The development of *Pseudomonas*-based microbial mixtures that was based on the beneficial properties of the individual components was sometimes successful, even without taking into account the possible lack of compatibility between the strains. For example, in a study conducted by Emami *et al*. (2018), a rhizospheric-endophytic mixed bacterial inoculant of two *Pseudomonas* strains with multi PGP traits was constructed, without carrying out any compatibility tests. Its application clearly increased plant biomass and micronutrient assimilation into grain of wheat compared to single strain inoculation under greenhouse conditions. Emami *et al.* (2019) suggested that co-inoculation of eight bacterial strains from different taxa (*Pseudomonas, Bacillus, Stenotrophomonas, Serratia*, *Nocardia* and *Microbacterium*) having multiple PGP traits, increased plant growth rather than single bacterial inoculation. In another experiment, when plant growth promoting *Pseudomonas* strains WCS417r and SS101 were co-inoculated as a mixture on *Arabidopsis thaliana* Col-0 roots, the density of Ps. WCS417r was 44 times higher than that of Pf. SS101 (Pangesti *et al*., 2017). The mixed inoculation reduced shoot fresh weight compared to single inoculation of WCS417r, whereas there was no effect on root fresh weight compared to single applications. Interestingly, the two strains were also found *in vitro* incompatible. Couillerot *et al.* (2011) reported *in vitro* incompatibility between *Azospirillum brasilense* Sp245 and *P. fluorescens* F113 with the latter being the inhibitor. Co-inoculation of the mixture on wheat plants showed a phytostimulatory effect similar to single inoculations, but the authors concluded it may be due to the action of *P. fluorescens* F113 alone since cells of *A. brasilense* Sp245 were 10 times less abundant on the root. It seems that minimization of the antagonistic activity among the components in a synthetic multistrain mixture, may maximize the consistency of the beneficial effect, because the antagonistic strain tends to dominate rather quickly even in two-strain co-cultures or co-colonization competition assays (Foster and Bell, 2012; Pangesti *et al*., 2017). Thus, it is becoming clear that the PGP properties of the components of the microbial mixtures should be considered along with their compatibility.

Based on a large number of studies, *Pseudomonas*-based multistrain mixtures appear to have a consistently greater efficacy on improvement of plant growth and/or biological control than the single strains. A microbial mixture consisted of *in vitro* compatible

strains *P. fluorescens* PF1 and *A. brasilense* TNAU enhanced groundnut plant growth more efficiently than each single inoculation, depending on the type of application (Prasad and Subramanian, 2017). The interaction between *Pseudomonas* and *Azospirillum* taxa may be influenced by the species or even strains. Indeed, growth of *A. brasilense* strains is differentially inhibited or enhanced by distinct *P. fluorescens* strains (Maroniche *et al*., 2018), confirming this hypothesis. *In vitro* compatible PGPR *Pseudomonas fluorescens* FAP2 and *Bacillus licheniformis* B642, successfully colonized rhizosphere and rhizoplane of wheat seedlings individually and by co-inoculation, increasing plant growth parameters compared to control (Ansari and Ahmad, 2019). Co-inoculation with the combination of *P. fluorescens* compatible strains RE8 and RS111 gave significant disease suppression of *Fusarium* wilt of radish in comparison with combination of incompatible strains RE8 and RS111a in a potting soil bioassay (de Boer *et al*., 1999). Similarly, the introduction of three compatible *P. fluorescens* isolates Pf1, TDK1, and PY15 was very effective in controlling population of the root-feeding nematode *Meloidogyne graminicola* in a field trial (Seenivasan *et al*., 2012), as well as in controlling sheath rot *Sarocladium oryzae* in rice (Saravanakumar *et al*., 2009). Co-inoculation of salt-sensitive pepper plants with *Pseudomonas* strains that were compatible in the rhizosphere improved the plant physiological properties under salinity stress compared to single inoculation (Samaddar *et al*., 2019).

Combining strains with different modes of action may increase the likelihood of building a consistently effective mixture against plant pathogens (Ruano-Rosa *et al*., 2014). Agusti *et al.* (2011) selected two compatible P. *fluorescens* strains which differed in secondary metabolite production and found that dual inoculations lead to better control of *Phytophthora cactorum* in strawberry compared to single introductions, suggesting that the different mechanisms of action between strains may act complementary or synergistically. Co-inoculation of detached potato leaves with two compatible *Pseudomonas* strains, weakly interfering with each other's growth**,** which had complementary modes of action against *Phytophthora infestans* was particularly efficient as compared to single-strain inoculation (De Vrieze *et al*., 2018). Also, *in vitro* compatibility tests showed antagonism between certain strains of *Pseudomonas* spp. and plant beneficial fungal strains of *Trichoderma* spp., but also permitted the selection of compatible strains for the construction of mixtures that promoted plant health and growth compared to each strain alone (Mishra *et al*., 2013).

A literature survey revealed an increasing number of examples where plant inoculation with compatible strains' mixtures of *P. fluorescens* and plant mutualistic bacteria (Sundaramoorthy and Balabaskar, 2012; Sundaramoorthy *et al.,* 2012; Sundaramoorthy and Balabaskar, 2013; Rathi *et al*., 2015; Kumar *et al*., 2016; Sharma *et al*., 2018) or beneficial fungi including species of *Trichoderma* (Thilagavathi *et al*., 2007, Jain *et al*., 2012, 2013, 2014, 2015; Singh *et al*., 2013a, 2013b, 2014; Ruano-Rosa *et al*., 2014; Thakkar and Saraf, 2015; Chemelrotit *et al*., 2017; Patel *et al*., 2017; Yadav *et al*., 2017; Jambhulkar *et al*., 2018), *Beauveria* (Karthiba *et al*., 2010; Senthilraja *et al*., 2013), *Pochonia* (Siddiqui *et al*., 2003) and *Clonostachys* (Karlsson *et al*., 2015) showed better results than inoculation with individual strains or control treatment, under controlled and field conditions. Furthermore, co-inoculation of specific *Pseudomonas* strains that function as mycorrhiza helper bacteria (MHB) in combination with various arbuscular mucorrhiza fungi (AMF) promoted the growth of maize plants in field conditions better than single AM inoculation (Berta *et al*., 2014). Prior testing of compatibility among strains is more likely to lead to the construction of a successful mixture

Bacillus-based multistrain mixtures

Among PGPMs, strains of *Bacillus* are the most widely used as biopesticides and biofertilizers (Aloo *et al*., 2018). As discussed

above, it is reasonable to assume that multistrain mixtures based on them may function in synergistic and additive manner compared to single-strain inoculants. Researchers have successfully engineered effective *Bacillus*-based multistrain mixtures without taking into account the compatibility of their components. A multistrain mixture consisted of *B. subtilis* AR12, *B. subtilis* SM21, and *Chryseobacterium* sp. R89, was shown to be a promising biocontrol agent against various diseases including Ralstonia wilt, Phytophthora blight and Meloidogyne root-knot of pepper under greenhouse and field conditions (Liu *et al*., 2014). Zhang *et al.* (2010) evaluated the efficacy of several *Bacillus*-based mixtures constructed using a pool of 12 bacilli strains known for their capacity to suppress *Phytophthora* blight on squash. Certain combinations of PGPR strains applied further increased the efficacy of disease control against *Phytopthora capsici* relative to their individual application but the authors concluded that the effect of mixtures cannot be predicted just by the performance of individual strains.

Brewer and Larkin (2005) screened various combinations of field and commercial bacterial and fungal strains and indicated that co-inoculation of *B. subtilis* GB03 (Kodiak, Gustafson) and *Trichoderma virens* GL-21 (SoilGard, Certis) provided a somewhat better control of stem canker caused by *Rhizoctonia solani* on potatoes than each organism alone, thus suggesting that certain bacterial and fungal mixtures may provide some synergistic effect in biocontrol efficacy. The other combinations did not show the desirable effect. Furthermore, several studies have demonstrated that mixtures of *Bacillus* spp. and *Trichoderma* spp. increased plant growth or the biocontrol efficiency against fungal phytopathogens more than each organism alone (Jisha and Alagawadi, 1996; Yobo *et al*., 2011; Ali *et al*., 2018; Alamri *et al*., 2019). They demonstrated that only a small fraction of the engineered mixtures exerted a better effect in controlling blight than the individual strains. Treatment with commercial formulation Trisan (*T. harzianum* AP-001) and Larminar (*B. subtilis* AP-01), applied alone or in combination, suppressed bacterial wilt (*R. solanace*arum), damping-off (*Pythium aphanidermatum*) and frogeye leaf spot (*Cercospora nicotiana*) of tobacco and protected the plant more effectively compared to the individual products (Maketon *et al*., 2008). Treatment of tomato with a mixture of commercial product BioYield (*Bacillus* spp. GBO3 and IN937a) and *B. licheniformis* CECT5106 showed a far better effect on tomato growth parameters and protection against *R. solani* than BioYield alone or the individual strains suggesting that increasing the diversity of microbial mixture may enhance the efficacy of the *Bacillus*-based mixture (Domenech *et al*., 2006). The effect of four different PGPB strains, *B. subtilis* GB03 and FZB24, *Bacillus amyloliquefaciens* IN937a and *Bacillus pumilus* SE34, applied individually and in different combinations of dual mixtures revealed that only the combination of IN937a and GB03 strains provided a higher control efficacy against *Fusarium oxysporum* f. sp. *radicis-lycopersici* on tomato than the individual strains (Myresiotis *et al*., 2012). In the previous studies, data concerning the compatibility of the microbial strains used are not presented, suggesting that construction of effective *Bacillus*-based multistrain mixture can be possible, but only when appropriate combinations are used.

The issue of compatibility among the components of a *Bacillus*-based multistrain mixture was early realized by researchers, thoroughly discussed and gradually implemented in their studies (Jetiyanon *et al*., 2003; Kloepper *et al*., 2004). A combination of *Bacillus* spp. strains BB11 and FH17, showing compatibility in the rhizosphere, enhanced yield and increased bioconrol efficiency against Phytophthora blight of bell pepper better than single strain inoculations (Jiang *et al*., 2006). Seed treatments with a mixture of *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a, showing rhizosphere compatibility, exhibited a greater plant growth promotion and protection against pathogens than any of the individual components (Kokalis-Burelle *et al*., 2006; Ryu *et*

al., 2007). The two-strain combination of *Bacillus* spp. GBO3 and IN937a was selected for the development of the product BioYield by Gustafson (Dallas, TX).

Liu *et al*. (2016a, 2016b, 2017, 2018) engineered synthetic *Bacillus*-based mixtures taking into account the biological control and plant growth promoting activities of individual strains as well as their *in vitro* compatibility. As a result, all the synthetic mixtures consistently showed a better efficacy in exerting the desirable effect in an additive or synergistic manner. In another study, the mixture of compatible *B. amyloliquefaciens* strain BLB369, *B. subtilis* strain BLB277 and *Paenibacillus polymyxa* strain 267 has been shown to stimulate wheat seed germination and exhibit better efficacy in controlling head blight caused by *Fusarium graminearum* than treatments with the individual strains or mixtures of two-strain combination (Zalila-Kolsi *et al*., 2016). The combined application of three compatible (colonization levels of cotton stems were similar for each strain) biocontrol strains on cotton roots, *B. subtilis* YUPP-2, *P. polymixa* YUPP-8 and *Paenibacillus xylanilyticus* YUPP-12, revealed better effect in controlling *Verticillium dahliae* in cotton than their individual application (Yang *et al*., 2013). Wang *et al*. (2016) evaluated the effect of a bacterial mixture composed of compatible *Bacillus* and *Serratia* strains (*Bacillus cereus* AR156, *B. subtilis* SM21, and *Serratia* sp. XY21) on alleviating cold stress; treated tomato plants had a far better survival rate than control plants. The same microbial mixture (*B. cereus* AR156, *B. subtilis* SM21, and *Serratia* sp. XY21) has been reported to be an efficient eco-friendly tool to induce drought tolerance in cucumber plants (Wang *et al*., 2012). Treatment of soybean with the mixture of compatible bacteria *Bradyrhizobium japonicum* MN110 and *Bacillus megaterium* LNL6 exhibited an increase in nodule number in pots at 35 days after sowing compared to single inoculation of MN110 (Subramanian *et al*., 2015).

Multistrain mixtures combining compatible *Bacillus* spp. and beneficial fungi were also constructed and successfully implemented. Treatments of banana with a mixture consisting of compatible *F. oxysporum* strain 162 and *Bacillus firmus* provided an enhanced biological control of the nematode *Radopholus similis* as compared to inoculation with single strains (Mendoza and Sikora, 2009). Application of a compatible combination of *B. subtilis* MF352017 and *T. harzianum* controlled chickpea wilt caused by *Fusarium oxysporum* f. sp. *ciceris* and enhanced plant growth as compared to individual application (Zaim *et al*., 2018). Treatment with a combination of compatible *B. subtillis* ATCC 11774, *T. harzianum* and *Trichoderma koningii* suppressed the development of potato stem canker as well as promoted growth and yield (Ali *et al*., 2018). Combinations of compatible *B. subtilis* and *Beauveria bassiana* have been successfully used for the control of wilt disease and fruit borer in tomato plants, broadening the range of the beneficial fungi that can be used for preparing *Bacillus*-based compatible mixtures (Prabhukarthikeyan *et al*., 2013). In another study, *B. pumilus* INR7 and *Rhizophagus* sp. were found to be compatible with each other. Combined application of INR7 and mycorrhiza not only suppressed plant disease caused by *R. solani* but also improved common bean dry weight either in simultaneous or delayed pathogen inoculation (Hussein *et al*., 2018).

On the contrary, application of commercial formulations of Serenade (*B. subtilis*) and Trianum (*T. harzianum T22*) or Sentinel (*T. atroviride* LC52) applied simultaneously or sequentially did not improve disease control compared to single application (Xu *et al*., 2010). The BCAs *B. amyloliquefaciens* CPA28 and *Penicillium frequentans* strain 909 (Pf909) in a mixture were less effective in controlling stone fruit brown rot caused by *Monilinia* spp. compared to their individual application. *P. frequentans* and *B. amyloliquefaciens* could not be combined because bacteria inhibited the germination and growth of *P. frequentans*. Furthermore, *B. amyloliquefaciens* outcompetes *P. frequentans* once applied on fruit surface (Guijarro *et al*., 2018). In the study of Thilagavathi *et*

al. (2017) mixture of incompatible *B. subtilis* Bs16 with *Trichoderma viride* strains Tv1 and/or Tv13, had the same or less effect on inhibition of *Macrophomina phaseolina* and produced greengram plants with a lower vigour index and germination percentage relative to their individual application. *Bacillus* species show strong antagonistic activity against other beneficial bacteria (Simoes *et al*., 2007) and fungi (Kim *et al*., 2008; Fuga *et al*., 2016), thus making the prior examination of compatibility a necessary step for the construction of an effective *Bacillus*-based mixture.

Fungal mixtures

Several studies have demonstrated that treatment of plants with mixtures of endophytic fungi have improved plant growth and health (Lugtenberg *et al*., 2016; Kashyap *et al*., 2017). Abundant endophytic fungi isolates applied to their own host or different hosts as a mixture significantly reduced disease symptoms by fungal pathogens, suggesting that endophytes suppress growth of invading pathogens either directly or indirectly (Arnold *et al*., 2003). A mixture of endophytic fungi isolated from wild barley effectively suppressed the seed-borne infections in a barley cultivar (Murphy *et al*., 2015). A fungal endophyte consortium consistently improved barley grain yield over several seasons under a variety of chemical fertilizer inputs and low seasonal rainfall (Murphy *et al*., 2017). Intra- or interspecies fungal consortia consisting of *Clonostachys, Beauveria, Metarhizium* or *Trichoderma* spp. are known to contribute to plant growth and health as biopesticides, biofertilizers, biostimulants and inducers of natural resistance to biotic and abiotic stress (Krauss and Soberanis, 2001; García *et al*., 2003; Hidalgo *et al*., 2003; Cota *et al*., 2008; Kapongo *et al*., 2008; Keyser *et al*., 2015; Chirino-Valle *et al*., 2016; Ren *et al*., 2016). However, the construction of the microbial mixtures was based on the effectiveness of each single isolate and the issue of compatibility among the isolates was not considered.

Inter- and intraspecies incompatibility among beneficial fungal isolates is quite often found (Reaves and Crawford, 1994; Krauss *et al*., 2004; Ruano-Rosa and López-Herrera, 2009; ten Hoopen *et al*., 2010; Krauss *et al*., 2013). Thus, antagonistic interactions between beneficial fungal strains could occur and decrease the efficacy of the treatment. Evaluation of *in vitro* interactions between *Clonostachys* and *Trichoderma* isolates revealed the dominant antagonistic activity of *Clonostachys* over *Trichoderma* strains suggesting that these two mycoparasites may be incompatible (Krauss *et al*., 2013). Co-inoculation of a mixture (1:10) of *Clonostachys rosea* and *Trichoderma* spp. on cocoa pods, temporarily suppressed *C. rosea*, whereas two weeks after application, *C. rosea* was the dominant and persistent pod colonizer (Krauss *et al*., 2013). However, these interactions may not be always antagonistic.

A mixture of *C. rosea* and *B. bassiana* (1:20) applied to flowers and leaves of tomato vectored by bees reduced significantly both grey mold and the insect pest (whitefly) suggesting that some kind of compatibility between these fungal species may occur under natural conditions (Kapongo *et al*., 2008). Application of a mixture of two compatible *C. rosea* isolates (Cr1 and Cr2) reduced the infection of cowpea seedlings by *Macrophomina phaseolina* in a pot experiment more efficiently, as well as resulted in higher yields compared to single-strain application (Ndiaye *et al*., 2010). Combinations of compatible *Trichoderma* isolates revealed that *most of the mixtures performed more efficiently in* controlling avocado white root rot *than the single application of BCAs* (Ruano-Rosa and López-Herrera, 2009). Also, the majority of the combinations of four compatible *Trichoderma* isolates were more effective in controlling postharvest crown rot of banana than a single isolate (Sangeetha *et al*., 2009). Mendoza and Sikora (2009) demonstrated that the combination of two compatible beneficial fungi, a nematode-antagonistic endophyte (*Fusarium oxysporum* strain 162) and an egg pathogen-

ic fungus (*Paecilomyces lilacinus* strain 251) were more effective in controlling *Radopholus similis* on banana than any antagonist applied alone.

Are the commercial multistrain mixtures consisted of compatible strains?

Currently, the majority of the PGPMs marketed as biopesticides, biofertilizers and biostimulants are comprised of a single strain, according to the label. However, bacterial and/or fungal multistrain mixtures are gradually becoming popular (Woo *et al*., 2014; Woo and Pepe, 2018), indicating a general shift in replacing the single strain inoculants. This shift is reflected in the increasing number of research publications, as discussed above, the boosting of patent files depositions and the interest of several companies in developing and launching multistrain microbial mixtures.

A number of companies are ready to launch multistrain mixtures into the market. An example is biofungicidal seed treatment Velondis Extra (BASF) containing *B. subtilis* strain BU1814 and *B. amyloliquefaciens* strain MBI 600 as a mixture. Another example is the combination of the rhizobia inoculant Nodulator (*Bradyrhizobium japonicum)* with the biofungicide Velondis Flex (*B. subtilis* strain BU1814) under the name Nodulator Duo

(https://agrow.agribusinessintelligence.informa.com/-/media/agri/agrow/ag-market-reviews pdfs/supplements/agrow_biologicals_2017_online.pdf).

Microbial multistrain mixtures developed by BioConsortia are in second or third year field trials for drought tolerance, nutrient use efficiency and yield improvement in stressed and standard agronomic conditions, while some new consortia for biofungicide activity are moving into their first year of field trials (https://agrow.agribusinessintelligence.informa.com/-/media/agri/agrow/ag-market-reviews-pdfs/supplements/agrow_biologicals_2017_online.pdf).

Recently, the Canadian authorities granted registration to Rootwin Plus-S, a combination of *Bradyrhizobium* spp. and *Trichoderma* spp., specifically to aid the soybean crop with rhizobium nodulation and to stimulate a healthy root system (https://www.andermattbiocontrol.com/).

Syngenta Agrochemical Company has launched the biofungicide Tellus (*Trichoderma asperellum* and *T. gamsii*) licensed from Italian company Isagro (https://agrow.agribusinessintelligence.informa.com/AG002647/Syngenta-presents-Tellus-biofungicide-in-Spain).

Monsanto BioAg in a new product, TagTeam, combines a rhizobial inoculant with the phosphorus solubilising fungus *Penicillium bilaiae* (O' Callaghan, 2016).

Adaptive Symbiotic Technologies have developed several fungal mixtures conferring tolerance to abiotic stresses (http://www.adaptivesymbiotictechnologies.com/products.html).

Bio Innovation AB filed a patent for the combination of antagonists *T. virens* isolate ATCC58678 and *B. subtilis* var. *amyloliquefaciens* strain FZB24 (https://patents.google.com/patent/CA2485796C/en). Another product, marketed under the trade name QuickRoots, contains a patented combination of the bacterium *B. amyloliquefaciens* and the fungus *T. virens*. The combination enhances the bioavailability of nitrogen, phosphorus and potassium in the soil resulting in expanded root volume and subsequent potential of enhanced yield (Parnell *et al*., 2016).

The Brazilian Ministry of Agriculture, Livestock and Supply has already issued the registration for the new multistrain mixture Shocker, recommended for the control of diseases, such as rhizoctoniosis and white mold, which mainly attack soy, coffee, cotton and minor crops. Shocker is composed of the bacteria *B. amyloliquefaciens* strain CPQBA 040-11DRM 01 and *B. amyloliquefaciens* strain CPQBA 040-11RRM 04 (http://news.agropages.com/News/NewsDetail---29634.htm).

Conclusion

The application of Plant Growth Promoting Microorganisms (PGPMs) or Plant Probiotics (PPs) as plant inoculants represents an environmentally friendly option for the reduction of chemical fertilizers and pesticides overuse. In general, synthetic microbial multistrain mixtures show better effect in promoting plant growth and suppressing plant disease compared to individual strains. Selection of the components is usually based on their individual plant growth promoting traits, not taking into account their possible antagonistic interaction. It seems, however, that the major issue of compatibility among the strains should be considered in the process of designing a mixture. Minimizing their antagonism may lead to a more consistent mixture, since they will not interfere with each other's growth and colonization capacity. Construction of even a dual strain successful mixture consisting of compatible components is not an easy task; nevertheless, it is an achievable one. Well-designed synthetic consortia of microbes can greatly increase the plant yield or control of plant pathogens in an environmentally sustainable way.

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ΑΡΘΡΟ ΑΝΑΣΚΟΠΗΣΗΣ

Σύγκριση μικροβιακών εμβολίων που προάγουν την ανάπτυξη των φυτών αποτελούμενων από μονά ή/και πολλαπλά στελέχη μικροοργανισμών – Το ζήτημα της συμβατότητας

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Περίληψη Οι μικροοργανισμοί που προάγουν την ανάπτυξη των φυτών (Plant Growth Promoting Microbes) ή οι φυτικοί προβιοτικοί μικροοργανισμοί, αποτελούν μια ιδιαίτερα υποσχόμενη λύση για την αειφόρο γεωργία. Η άποψη ότι ο εμβολιασμός φυτών με μίγματα που περιέχουν τους εν λόγω μικροροganισμούς είναι αποτελεσματικότερος, σε σχέση με την εφαρμογή μεμονωμένων στελεχών τους, χρονολογείται από την ανακάλυψη των ριζοβακτηρίων που επάγουν την ανάπτυξη των φυτών και ανακτά έδαφος στις μέρες μας. Ο αυξανόμενος αριθμός επιστημονικών δημοσιεύσεων για τη θετική επίδραση των μικροβιακών μιγμάτων στην προαγωγή της ανάπτυξης των φυτών, στον έλεγχο των παθογόνων των φυτών καθώς και στην επαγωγή αντοχής υπό αβιοτική καταπόνηση, επιβεβαιώνει την παγκόσμια τάση εφαρμογής μικροβιακών εμβολίων. Η συνεχής κατάθεση ευρεσιτεχνιών καθώς και η διαθεσιμότητα εμπορικών σκευασμάτων που αφορούν σε βιοπροστατευτικά ή/και βιοδιεγερτικά μίγματα πολλαπλών στελεχών, επίσης ενισχύουν την τάση αυτή. Ένα σημαντικό ζήτημα για το σχεδιασμό ενός πιο αποτελεσματικού και σταθερού συνθετικού μίγματος πολλαπλών στελεχών, αποτελεί η συμβατότητα μεταξύ των μικροβίων. Το παρόν άρθρο ανασκόπησης παρέχει μια διεξοδική βιβλιογραφική έρευνα που υποστηρίζει την άποψη ότι η μεταχείριση των φυτών με μίγματα πολλαπλών στελεχών, συμβατά μεταξύ τους, συμβάλει στην αποδοτικότερη ανάπτυξη και υγεία των φυτών σε σχέση με την εφαρμογή μεμονωμένων στελεχών. Η μελέτη μας επικεντρώνεται σε μίγματα πολλαπλών στελεχών που έχουν ως βάση στελέχη του γένους *Pseudomonas* και *Bacillus* καθώς και στελέχη ωφέλιμων μυκήτων, ενώ γίνεται αναφορά σε διαθέσιμα εμπορικά σκευάσματα.

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Exploring environmental determinants of Fusarium wilt occurrence on banana in South Central Mindanao, Philippines

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Summary This study used Maximum Entropy (MaxEnt) to explore potential environmental determinants of Fusarium wilt occurrence on banana in south-central part of the Philippines. Different variables representing topographic, bioclimatic, and edaphic features of an area were tested against data of Fusarium wilt occurrence. Based on the results, precipitation during the driest month, precipitation during the wettest month, precipitation of the warmest quarter, slope, and elevation were the most important variables for predicting the probability of Fusarium wilt occurrence on banana. Results also suggest that among the variables tested, precipitation had the major contribution to the occurrence of Fusarium wilt.

Additional keywords: Climate, MaxEnt, Panama disease, topography

Introduction

Banana (*Musa* sp.) is an important subsistence food and high value commercial crop in the world (Ghag *et al*., 2015; Ravi and Vaganan, 2016). Banana is grown in more than 120 countries and its cultivation and related activities provide livelihood to many families in Africa, Asia, and Latin America (Roux *et al*., 2008; Ghag *et al*., 2015). In the Philippines, banana is the top fruit crop grown and a consistent dollar earner for the country (Solpot *et al*., 2016). In 2015, around 0.44 million hectares were planted with banana resulting in more than 9 million metric tons of produce with an estimated value of around USD 2.7 billion (PSA, 2017). Cavendish cultivars (50%) have the largest contribution to the country's banana production followed by Cardava (28%) and Lakatan (10%) cultivars (Solpot *et al*., 2016). Top banana producing areas are mostly found in the southern part of the Philippines (Solpot *et al*., 2016).

Fusarium wilt, also known as Panama disease, is an important disease of banana that has devastated thousands of hectares of plantations worldwide (Ploetz, 2006, 2015a, 2015b; Ghag *et al*., 2015). Fusarium wilt is a soil-borne disease that causes wilt and severe die back to banana plant and can persist in the soil for at least 30 years (Stover, 1962; Cook *et al*., 2015). The disease is caused by the fungal pathogen *Fusarium oxysporum* f. sp. *cubense* (Foc) (Ploetz, 2006; 2015a; 2015b; Ghag *et al*., 2015). To enter the roots, Foc invades the epidermal cells on the root cap and elongation zone, and the small wounds along the lateral root base (Li *et al*., 2011; Pattison *et al*., 2014). Then, Foc proceeds to the vascular system causing the disease (Li *et al*., 2011; Pattison *et al*., 2014). Once in the vascular tissues, the pathogen disrupts the water translocation causing wilting symptoms, such as drooping foliage and leaf chlorosis that start from the lower to the upper leaves, resulting in plant necro-

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sis and death (Li *et al*., 2011; Pattison *et al*., 2014; Ploetz, 2015a). In Australia alone, Cook *et al*. (2015) estimated an annual loss of more than 138 million USD to the banana industry due to Fusarium wilt.

Despite numerous studies and reviews on the epidemiology and management of Fusarium wilt of banana, there is limited literature on the environmental factors that affect its incidence and severity rates (Ploetz, 2006, 2015a, 2015b; Pattison *et al*., 2014; Ghag *et al*., 2015). For example, Pattison *et al*. (2014) found that Fusarium wilt expression is a function of water stress (deficit and excess) and heat unit requirement of banana. Deltour *et al*. (2017) showed that the higher the clay content, pH, and electric conductivity in soil, the lesser severity of Fusarium wilt. Meanwhile, according to Perez-Vicente *et al*. (2014), severe infection is observed during the warmer and wet months of the year. Karangwa *et al*. (2016) reported that Fusarium wilt incidence and distribution is associated with elevation.

Maximum Entropy (MaxEnt) is a general-purpose machine learning method that has a simple and precise mathematical formulation well suited for modeling the geographic distribution of species using presence-only data (Phillips *et al*., 2006). According to Phillips *et al*. (2006), MaxEnt estimates a target probability distribution based on distribution of maximum entropy (i.e. closest to uniform), subject to a set of constraints related to incomplete information regarding the target distribution. For example, the pixels of a study area constitute the MaxEnt probability distribution while the pixels with occurrence records are the sampling points, and the different environmental variables or covariates (e.g. climate, elevation, soil, vegetation) represent the features (Phillips *et al*., 2006). MaxEnt also uses background points (points where presence or absence is unmeasured) that contrast against the occurrence points (presence locations) to estimate probability of occurrence (Merow *et al*., 2013). According to Phillips *et al*. (2006), MaxEnt has many advantages compared with other modeling methods. MaxEnt requires only presence data and environmental variables for the whole study area. Also, it can use both continuous and categorical data. In addition, it has efficient deterministic algorithms and performs better than other methods even with small sample size (Wisz *et al*., 2008). Detailed description of MaxEnt can be found in Phillips *et al*., (2006), Elith *et al*. (2011), Merow *et al*. (2013).

MaxEnt (Phillips *et al*., 2006) is the most popular software package used for modeling species geographic distribution using presence-only data (Elith *et al*., 2011; Merow *et al*., 2013). According to Elith *et al*. (2011), since MaxEnt became available in 2004, it has been extensively utilized for species distribution modeling that aims at finding correlates of species occurrence, mapping current and future species distribution across many ecological, evolutionary, conservation, and biosecurity applications. In fact, since 2006, there are thousands of publications about the application of MaxEnt (Merow *et al*., 2013). In plant pathology, several studies have used MaxEnt to identify environmental determinants and map potential distribution of plant diseases and their vectors (e.g. Wyckhuys *et al*., 2012; Bosso *et al*., 2016; Galdino *et al*., 2016; Narouei-Khandan *et al*., 2016; Shimwela *et al*., 2016; Vallejo Pérez *et al*., 2017). This study aims to identify environmental factors (i.e. topographic, edaphic, and bioclimatic) favoring Fusarium wilt infection of banana in South Central Mindanao, Philippines *via* the MaxEnt-modeling approach in order to develop a model for predicting disease occurrence and assessing the risk.

Meterials and Methods

Presence-Only Data

Presence-only data were adapted from the earlier study by Solpot *et al*. (2016) in which Foc-infected plant samples (75 points) were collected from different provinces in the south-central part of the country (Fig. 1). Plants that showed typical external and internal symptoms of Foc, such as wilting, yellowing of leaves, and pseudostem and corm

discoloration were collected. Geographic coordinates of sampled plants were tagged using a global positioning system (GPS) receiver. Foc was isolated using the tissue plating technique. Full details of sampling and analysis of Foc sampled plants can be found in Solpot *et al.* (2016). Table 1 summarizes the number of Foc isolates by location and banana cultivar collected from the study area (Solpot *et al.*, 2016).

Environmental Data

Environmental data used in the study included topographic, edaphic, and climatic variables (Table 2). Topographic data included elevation, slope, and aspect. Elevation data of the study area at 1 km x 1 km spatial resolution was extracted from shuttle radar topography mission (SRTM) (Farr *et al.*, 2007). Slope and aspect were derived from elevation data using terrain function of R software (Ihaka and Gentleman, 1996; R Core Team, 2014) raster package (Hijmans, 2014). Meanwhile, 1km x 1km spatial resolution soil data (i.e. pH, CEC, organic carbon content, % clay, % silt, and % sand) of the study area were downloaded from the SoilGrids database at 250 m resolution (Hengl *et al.*, 2017). Bioclimatic data were derived from downscaled (1 km x 1 km) Climate Research Unit Time Series (CRU TS) data (Harris *et al.*, 2014) for the Philippines (Salvacion *et al.*, 2018). Ten bioclimatic variables (Booth *et al.*, 2014) were used in this study.

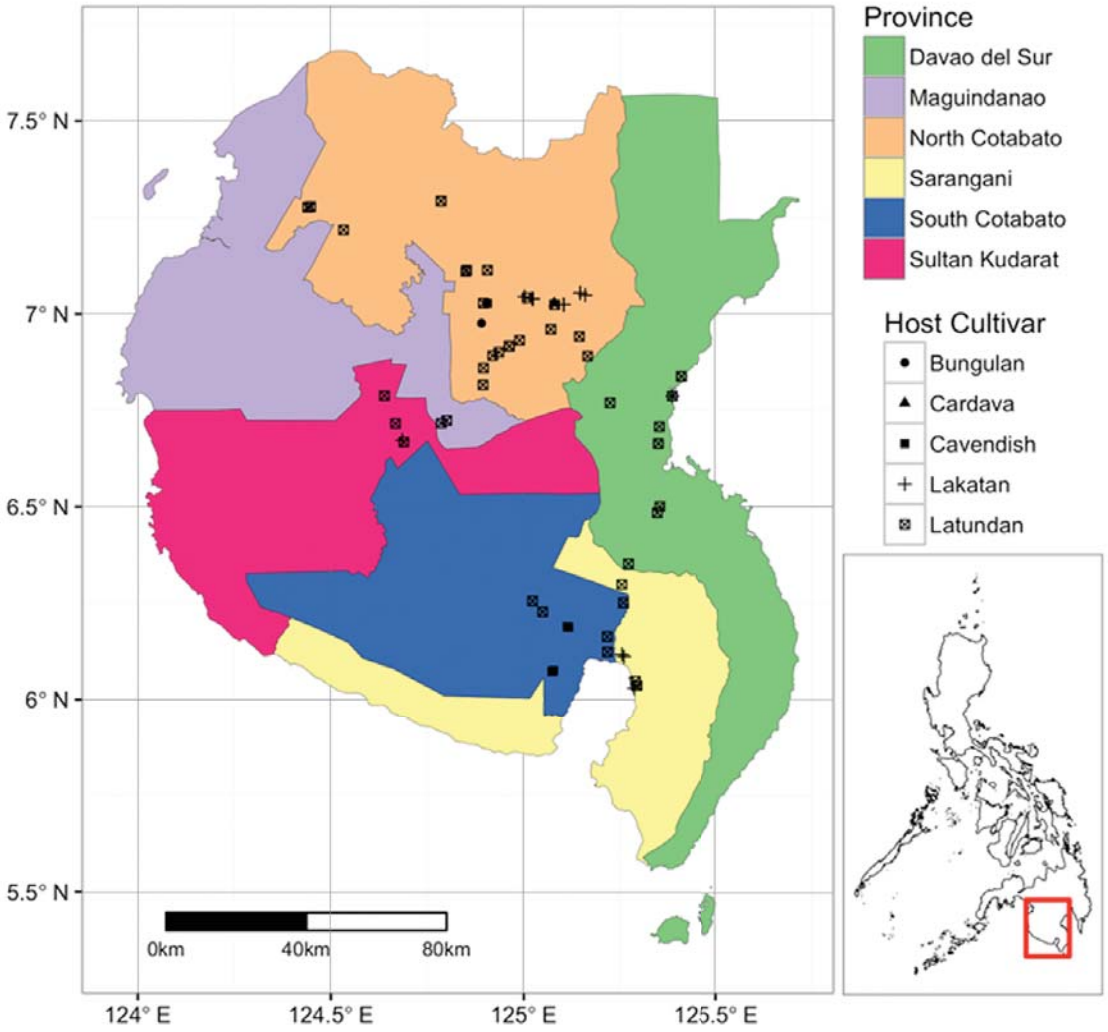

Figure 1. Location map of *Fusarium oxysporum* f. sp. *cubense* (Foc) and banana cultivars sampling points in south-central Mindanao, Philippines.

MaxEnt Modeling

Presence-only data were split (80:20) into training (60 points) and test/validation data (15 points) sets. Also, background data (1000 points) were generated randomly across the study area. A step-wise model building was also adapted by removing variables with permutation importance less than 5% (Heumann *et al*., 2011; Kalle *et al*., 2013; Zeng *et al*., 2016). Permutation importance measures how the model depends on the variable (Galdino *et al*., 2016). In this

Table 1. Number of *Fusarium oxysporum* f. sp *cubense* (Foc) isolates per host cultivar collected in different provinces in south-central Philippines.

Host Cultivar	Province						Total
	North Cotabato	South Cotabato	Saranggani	Davao Del Sur	Sultan Kudarat	Maguindanao	
Latundan (AAB)	23	5	6	5	3	2	44
Lakatan (AAA)	12	0	3	1	1	0	17
Cavendish (AAA)	1	11	0	0	0	0	12
Cardaba (ABB)	1	0	0	0	0	0	1
Bungulan (AAA)	1	0	0	0	0	0	1
Total	38	16	9	6	4	2	75

Table 2. Environmental data for modeling Fusarium wilt in banana.

Variable	Description	Unit
Topographic		
Elevation	Elevation	masl
Slope	Slope	degrees
Aspect	Aspect or slope direction	-
Edaphic		
Soil pH	Soil pH	pH units
CEC	Cation Exchange Capacity	cmolc/kg
Organic carbon content	Organic carbon content	g/kg
% Clay	Clay content (0-2 micro meter) mass fraction	%
% Silt	Silt content (2-50 micro meter) mass fraction	%
% Sand	Sand content (50-2000 micro meter) mass fraction	%
Climatic		
Bio 1	Annual Mean Temperature	°C
Bio 5	Maximum Temperature of Warmest Month	°C
Bio 6	Minimum Temperature of Coldest Month	°C
Bio 8	Mean Temperature of Wettest Quarter	°C
Bio 9	Mean Temperature of Driest Quarter	°C
Bio 12	Annual Precipitation	mm
Bio 13	Precipitation of Wettest Month	mm
Bio 14	Precipitation of Driest Month	mm
Bio 18	Precipitation of Warmest Quarter	mm
Bio 19	Precipitation of Coldest Quarter	mm

study, MaxEnt package (Phillips *et al.*, 2006; 2018) was run via R *dismo* package (Hijmans *et al.*, 2016) using default settings.

Model Validation

Area under the curve (AUC) was calculated for both training and test data sets to determine the model's predictive power and potential over-fitting (Elith *et al.*, 2011; Merow *et al.*, 2013; Bosso *et al.*, 2016). According to Rödder *et al.* (2009), AUC ranges from 0.5 (no predictive ability) to 1.0 (perfect prediction). An AUC value of 0.7-0.8 means that the model is useable, a value of 0.8-0.9 indicates good performance, and a value of 0.9-1.0 signifies very good predictive power (Rödder *et al.*, 2009). Meanwhile, other measures (Table 3) of model's predictive accuracy were calculated using the test data points for model validation (Allouche *et al.*, 2006). According to Allouche *et al.* (2006), true skill statistic (TSS) values range from -1 to +1, where values of zero or less indicate poor performance and +1 indicates perfect agreement.

Results

Step-wise model selection and validation

Only five out of the 19 variables in the initial model were left in the final model (Table 4). These variables included slope, elevation, precipitation on the driest month, precipitation on the wettest month, and precipitation on the warmest quarter. Precipitation during the wettest month had the highest permutation importance (26.1%) followed by slope (24.9), while precipitation during the warmest quarter had the lowest (12%). The AUC for the training and test data was 0.89 and 0.88, respectively. This suggests that the final model performed very well with respect to the training and test data (Elith, 2000; Rödder *et al.*, 2009; Abdullah *et al.*, 2017). These results were further confirmed by the different measures of model accuracy (Allouche *et al.*, 2006) in Table 5 using validation data points. Figure 2 shows the predicted presence of Fusarium wilt along with training (Fig. 2a) and validation data points (Fig. 2b).

Table 3. Measure of predictive accuracy of the model (Source: Allouche *et. al*, 2006).

Measure	Formula	Description
Overall accuracy	$\frac{a+d}{n}$	Rate of correctly predicted presence and absence data
Sensitivity	$\frac{a}{a+c}$	Probability that the model will correctly classify a presence data
Specificity	$\frac{d}{b+d}$	Probability that the model will correctly classify a absence data
Kappa	$\frac{\left(\frac{a+d}{n}\right) - \frac{(a+b)(a+c)+(c+d)(d+b)}{n^2}}{1 - \frac{(a+b)(a+c)+(c+d)(d+b)}{n^2}}$	Kappa and TSS normalize the overall accuracy by the accuracy due chance alone
True Skill Statistic (TSS)	sensitivity + specificity – 1	

where: *a - number of "presence" points for which was correctly predicted by the model*
b - number of "absence" points which the model predicted as "presence"
c - number of "presence" points which the model predicted as "absent"
d - number of "absence" points for which was correctly predicted by the model
n – a+b+c+d

Table 4. Permutation importance of environmental variables included in the final model.

Variable	Permutation Importance (%)
Climatic	
Precipitation of Wettest Month	26.1
Precipitation of Driest Month	21.2
Precipitation of Warmest Quarter	12.0
Topographic	
Slope	24.9
Elevation	15.8

Table 5. Calculated measure of predictive accuracy of the final model.

Measure	Value
Overall accuracy	0.70
Sensitivity	1
Specificity	0.68
Kappa	0.21
True Skill Statistic (TSS)	0.68

Environmental Responses

In terms of bioclimatic variables, similar behavior was observed for the effect of precipitation during the driest (Fig. 3a) and wettest (Fig. 3b) months of the year. Higher probability was estimated for lower values of precipitation for these months (Fig. 4). For the wettest month of the year, the highest probability of occurrence (0.95) was calculated for monthly precipitation of 100 mm and eventually decreased to zero starting at monthly precipitation of 332 mm (Fig. 4a). For the driest month of the year, the highest probability of occurrence (0.46) was calculated at 43 mm of precipitation and decreased to zero starting at 120 mm monthly precipitation (Fig. 4b). On the other hand, the probability of Fusarium wilt occurrence showed a different response to precipitation during the warmest quarter (Fig. 4c). Higher probability of occurrence was observed on higher precipitation amount. More specifically, the highest probability (0.99) was estimated for quarterly precipitation of more than 839 mm and the lowest (0.01) for quarterly precipitation of less than 207 mm (Fig. 4c). Figure 5 shows the warmest quarter corresponding the sampling locations of Fusarium wilt occurrence.

Regarding topographic variables, higher probabilities of Fusarium wilt occurrence were estimated at lower slope and elevation values (Fig. 6). This means that higher chance of Fusarium wilt infection is expected in flat and lowland areas compared to sloping and upland ones. The highest probability of Fusarium wilt (0.47) was estimated at 0.10° slope and decreased exponentially to zero starting at slope equal to 8.51° (Fig. 6a). With respect to elevation, the highest probability of Fusarium wilt occurrence (0.23) was observed at 40 meters above sea level (masl) and exponentially decreased to zero starting at 1108 masl (Fig. 6b).

Discussion

Results suggest that bioclimate (i.e. precipitation) is the major contributory factor on Fusarium wilt occurrence. Low (less than 120 mm) monthly precipitation during the driest and wettest month of the year also results to higher probability of occurrence. Conversely, higher probability of occurrence is expected for higher precipitation (greater than 800 mm) during the warmest quarters. Topography (slope and elevation) of the area also influences occurrence of the disease. The probability of Fusarium wilt occurrence is higher on flat areas (less than 8° of slope) and areas with low elevation (less than 40 masl).

The effect of precipitation and slope on the occurrence of Fusarium wilt of banana can be attributed to the response of Foc and banana plant to water availability or soil moisture. Low rainfall during the driest and wettest quarter can subject the banana plant to low moisture or water deficit stress condition making it highly susceptible to severe infection by the pathogen (Lee *et al*., 2004; Ghaemi *et al*., 2011; Pattison *et al*., 2014). Also, such conditions promote increased root colonization of tomato plants

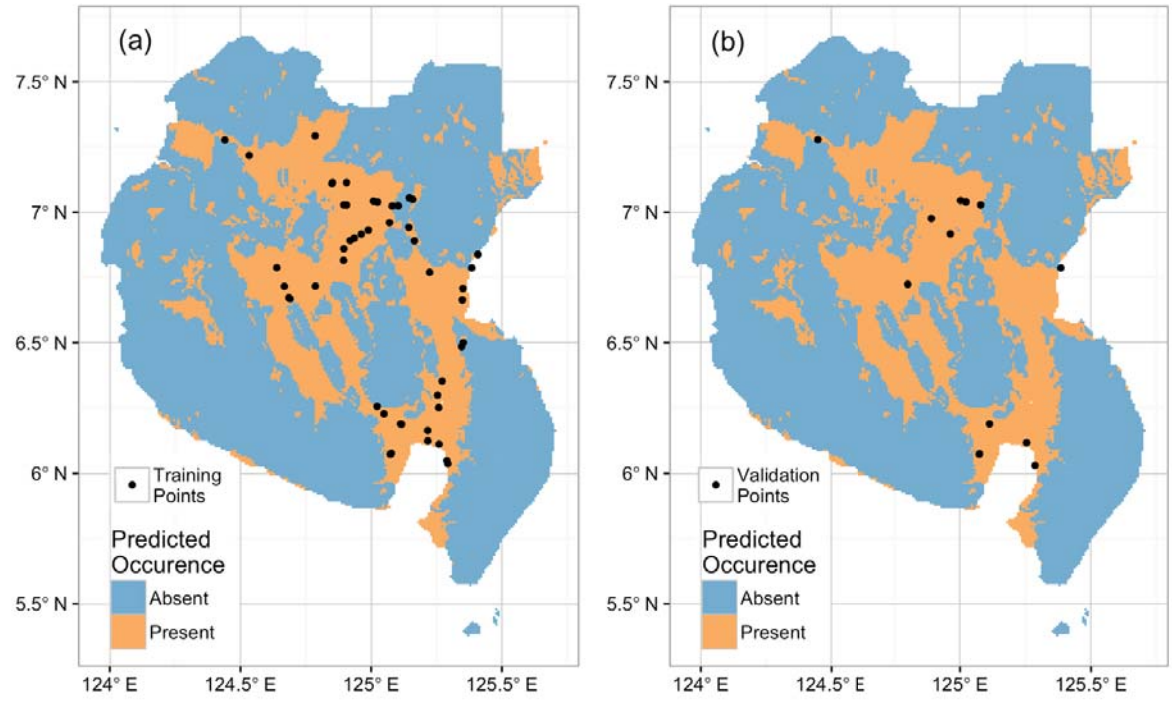

Figure 2. Predicted occurrence of *Fusarium oxysporum* f. sp. *cubense* (Foc) in south central Mindanao, Philippines, with (a) training, and (b) validation data points.

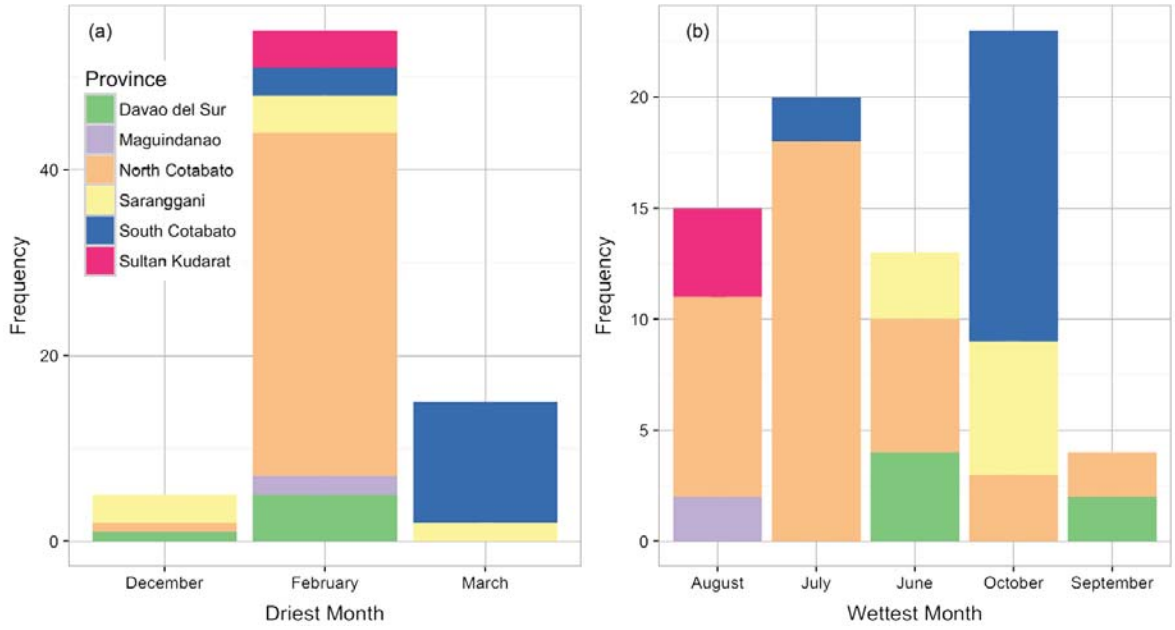

Figure 3. Driest (a) and wettest (b) months of each province in south-central Mindanao, Philippines, corresponding to *Fusarium oxysporum* f. sp. *cubense* (Foc) sampling points.

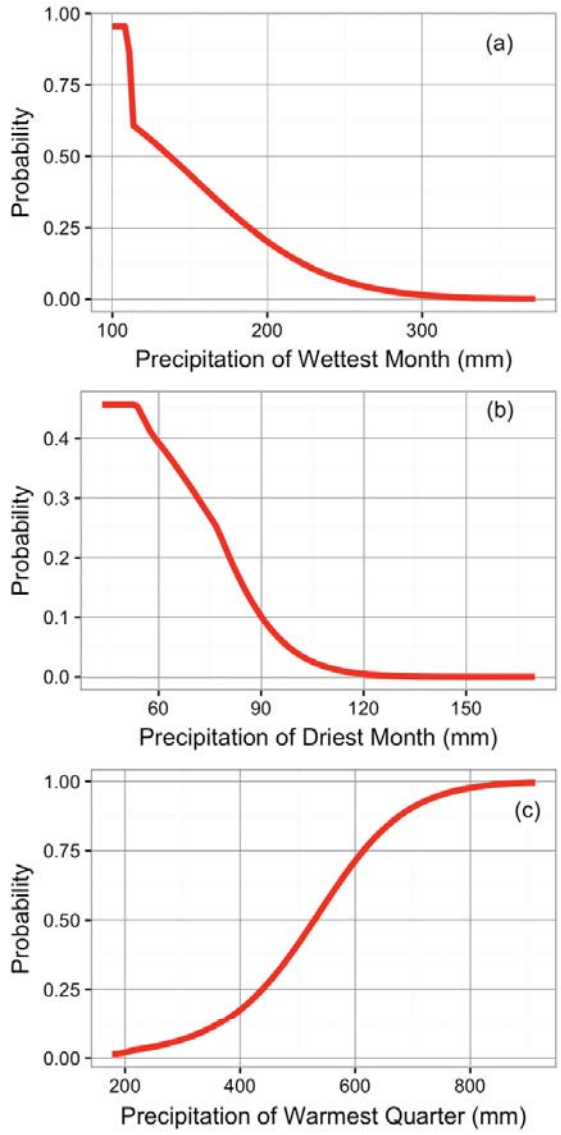

Figure 4. Response curve of Fusarium wilt (*Fusarium oxysporum* f. sp. *cubense)* occurrence with respect to precipitation on: (a) the wettest month; (b) the driest month, and (c) the warmest quarter in south-central Mindanao, Philippines.

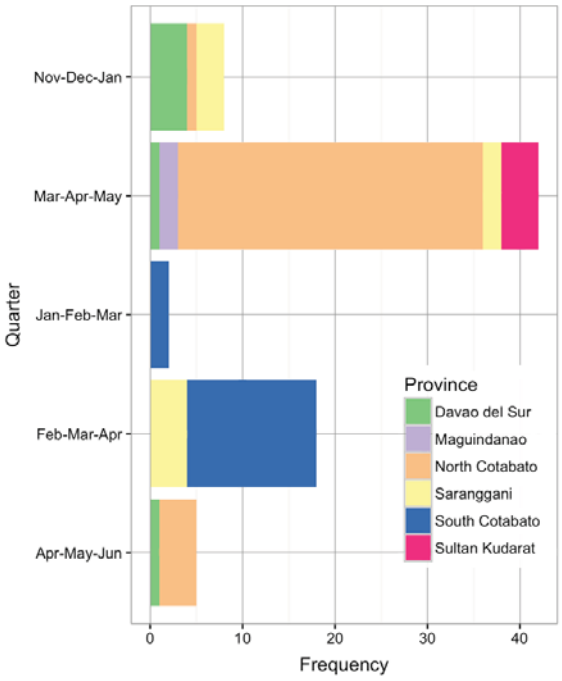

Figure 5. Warmest quarter of each province in south-central Mindanao, Philippines corresponding to *Fusarium oxysporum* f. sp. *cubense* (Foc) sampling points.

by *Fusarium oxysporum* f. sp. *lycopersici* (Ghaemi *et al*., 2011). Meanwhile, higher precipitation during the warmest quarter can result in higher probability of Fusarium wilt occurrence because such conditions (warm and wet) are conducive to severe infection of banana by the pathogen (Perez-Vicente *et al*., 2014). Also, higher rainfall can saturate soil producing anoxic conditions, which can enhance Foc root infection (Aguilar, 1998; Aguilar *et al*., 2000; Pattison *et al*., 2014).

Areas with flat to near flat topography tend to have relatively higher moisture availability compared to the sloping ones, thus providing optimum conditions for fungal growth (Stover, 1953; Salvacion, 2016). In addition, slope can affect different soil properties (Su *et al*., 2010), which may also affect Foc presence or abundance (Fu *et al*., 2016; Deltour *et al*., 2017). This could probably be the reason why soil variables in this study showed no significant effect on Fusarium wilt occurrence.

The influence of elevation on Fusarium wilt incidence observed in the present study was similar to that of previous studies elsewhere (Kangire *et al*., 2001; Karangwa *et al*., 2016). According to Karangwa *et al.* (2016), the effect of elevation on Fusarium wilt development may be due to the temperature variation as influenced by elevation. Fusarium wilt development is encouraged by higher temperatures at lower altitudes (Karangwa *et al*., 2016).

The results of this study corroborate to previous studies conducted elsewhere. Lee *et al.* (2004) observed higher severity of Fusarium wilt in sweet potato at precipitation lower than 80 mm during planting

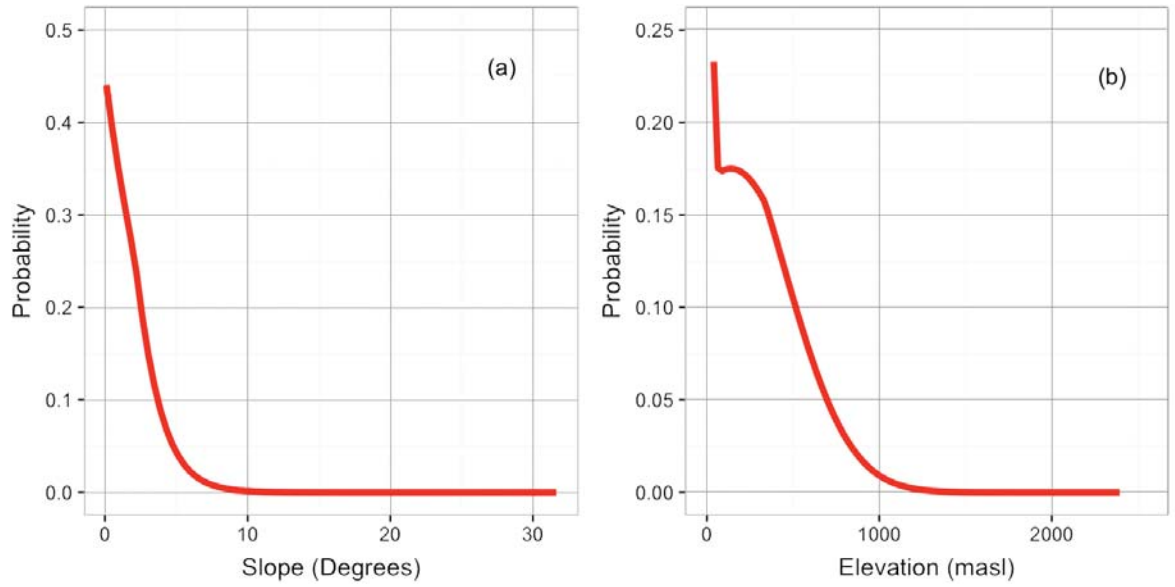

Figure 6. Response curve of Fusarium wilt (*Fusarium oxysporum* f. sp. *cubense*) occurrence with respect to: (a) slope, and (b) elevation (meters above sea level, masl) in south-central Mindanao, Philippines.

season. Fusarium wilt of sweet potato was higher in flat areas compared to that in areas situated in sloping sites (Lee *et al*., 2004). In Australia, Pattison *et al.* (2014) observed higher incidence of Fusarium wilt of banana during months with rainfall less than 100 mm and greater than 500 mm. Karagwa *et al.* (2016) observed higher incidence of Fusarium wilt infection on banana farms located at elevations less than 1600 masl in east and central Africa.

Models like MaxEnt also have uncertainties resulting from sampling bias, quality of occurrence data, spatial resolution of environmental data, spatial autocorrelation and species characteristics (Dormann *et al*., 2008; Jarnevich *et al*., 2015; Galdino *et al*., 2016). In the case of the present study, sampling was done based only on the reported cases of Fusarium wilt occurrence. In addition, spatial autocorrelation among sampling points and environmental variables was not considered in the model building. Furthermore, the environmental data used in the study has also uncertainties (Hijmans *et al*., 2005; Hengl *et al*., 2017; Salvacion, Macandog *et al*., 2018). Lastly, the resolution of the environmental data might also have impact on the final model (Gillingham *et al*., 2012; West *et al*., 2015, 2016). At present, there is no or limited high resolution and updated environmental data (e.g. topography, climate, soil) in the country. Therefore, caution is recommended in interpreting the results of this study. Also, other approaches to analyze spatially referenced disease data might have different results (Turechek and McRoberts, 2013; Galdino *et al*., 2016).

The information, such as the range of environmental conditions favoring occurrence of Foc on banana and the model derived in this study can be used as a preliminary tool to assess potential risk of disease occurrence in other parts of the country. In addition, since climate has a major role in Fusarium wilt occurrence, the model derived from this study can also be used to determine potential impact of climate change on disease presence in the country. Such information can help farmers, managers, and policy makers to have an informed decision on how to avoid or minimize losses due to Fusarium wilt of banana.

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Διερεύνηση των περιβαλλοντικών παραμέτρων που καθορίζουν την εμφάνιση της αδροφουζαρίωσης στη μπανάνα στο Νότιο Κεντρικό Μιντανάο, Φιλιππίνες

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Περίληψη Η παρούσα μελέτη χρησιμοποίησε τη μέθοδο Maximum Entropy (MaxEnt) για να διερευνήσει τις πιθανές περιβαλλοντικές παραμέτρους που καθορίζουν την εμφάνιση της αδροφουζαρίωσης στη μπανάνα (*Fusarium oxysporum* f. sp. *cubense*), στο νότιο-κεντρικό τμήμα των Φιλιππίνων. Ελέγχθηκαν διάφορες μεταβλητές που αντιστοιχούν σε τοπογραφικά, βιοκλιματικά και εδαφικά χαρακτηριστικά μιας περιοχής σε σχέση με τα δεδομένα εμφάνισης της αδροφουζαρίωσης. Με βάση τα αποτελέσματα, η βροχόπτωση κατά τη διάρκεια του ξηρότερου και υγρότερου μήνα, η βροχόπτωση κατά τη διάρκεια του θερμότερου τριμήνου του έτους, η κλίση του εδάφους και το υψόμετρο της περιοχής ήταν οι πιο σημαντικές μεταβλητές για την πρόβλεψη της πιθανότητας εμφάνισης της ασθένειας στη μπανάνα, με σημαντικότερη μεταβλητή τη βροχόπτωση.

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Diverse responses of old, modern and landraces of Syrian wheat genotypes to common root rot under field conditions

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Abstract The yield response of widely grown cultivars and landraces of Syrian wheat challenged with common root rot (CRR: *Cochliobolus sativus*) was measured by comparing plots with and without artificial inoculation under experimental conditions in two consecutive seasons. The results showed that response to CRR differed depending on the susceptibility levels of the wheat cultivars, and that the disease significantly ($P<0.05$) reduced grain yield, number of tillers and kernel weight. The diseased plants had fewer tillers which consequently reduced grain yield per plant. Yield losses of *Triticum durum* cultivars were higher than those of *Triticum aestivum*. In addition, the *T. durum* landrace Horani exhibited the best level of resistance to the disease, which indicates that this landrace might be a candidate donor for resistance in future breeding programmes. As CRR can dramatically reduce wheat grain yields under favorable conditions, management practices that reduce disease severity are highly recommended.

Additional keywords: *Cochliobolus sativus, Triticum aestivum, Triticum durum,* yield loss.

Introduction

Common root rot (CRR), caused by *Cochliobolus sativus* (Ito & Kurib.) Drechsl. ex Dast. [anamorph: *Bipolarissorokiniana* (Sacc. in Sorok.) Shoem.], is an economically important disease of barley, wheat and other small grains in semi-arid climates worldwide (McKay*et al*., 2018). CRR causes a brown to black discoloration of the subcrown internodes (SCIs) of wheat (*Triticum aestivum* L.), which is directly related to yield losses (Mathre *et al*., 2003; Fernandez Holzgang, 2009).

Although fungicides can reduce disease severity, the most effective and environmentally sound means of control is through the use of resistant cultivars (Kumar *et al*., 2002). Wheat interaction with CRR is genotype dependent (Fernandez and Jefferson, 2004) and affected by soil inoculum (Smiley *et al*., 2005). Therefore, prior to controlling CRR, the potential of this disease to cause losses in wheat growing areas should be evaluated.

The impact of CRR on the crop (wheat) is important because reductions in plant biomass are a measure of the combined effects of the disease on photosynthesis and other production processes (Fernandez and Conner, 2011). Therefore, this study was carried out to evaluate wheat yield responses to CRR under experimental conditions that are typical of a large part of the wheat-growing areas of western Asia.

Materials and Methods

Plant material

Ten most widely grown cultivars and landraces of Syrian wheat were used in the study. They included two *Triticum durum* landraces (Horani and Salamoni), four *Triticum aestivum* cultivars (Bouhouth4, Bouhouth6, Cham2 and Doma4), one *T. aestivum* introduced cultivar (Maksibak) and three *T. durum* cultivars (Bouhouth7, Cham3 and Doma1).

Seed inoculation

Nine isolates of *C. sativus,* selected on the basis of cultural and morphological characteristics and virulence (Arabi and Jawhar, 2002), were used. These isolates were obtained from subcrown internodes of bar-

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ley plants showing CRR symptoms. Each isolate was grown on potato dextrose agar (PDA, DIFCO, Detroit, MI, USA) for 10 days at 22 ±1°C in the dark. After 10-12 days, conidia were collected by flooding the plate with 10 mL of sterile distilled water and scraping the colony surface with a glass slide to dislodge the conidia. Equal volumes of conidial suspension of each isolate were mixed and filtered through a double layer of cheesecloth. The resulting conidial suspension was adjusted to 5 X 10^5 conidia/mL.

Experimental design

The trials were conducted at a site approximately 55 km south of Damascus for two consecutive years (2016-2017), under natural rainfed conditions (350mm annual rainfall). Seed inoculation was performed according to the method described by van Leur (1991), where, 30 g seeds of each cultivar were placed in a plastic Petri dish (12-cm in diameter) containing 10 g sterile neutralized peat, 40 ml spore suspension (5 × 10^5 condia/ml) and 8 drops of natural Arabic gum. Following thorough agitation for 1 min, the seeds were sown at 6 cm depth to promote long subcrown internodes (Kokko *et al*., 1995) in a randomized complete block design, with three replicate plots (1 m x1 m) separated with a 1-m wide border. Each plot consisted of five rows, 20 cm apart and with 50 seeds per row. Based on laboratory preliminary tests on PDA media, CRR-free seeds were used as controls.

Disease evaluation

Subcrown internodes (SCIs) were examined 8 weeks post-inoculation by measuring the percentage of SCIs surface showing CRR symptoms using a 0-5 scale, as described by Kokko *et al.* (1995), where 0 (resistant); 1 = HT (highly tolerant): small light brown lesions covering 1-10% of the SCI; 2 = T (tolerant): light brown lesions covering 11-25% of the SCI; 3 = MS (moderately susceptible): light brown/black lesions covering 26-40% of the SCI; 4 = S (susceptible): black lesions covering 41-75% of the SCI; 5 = HS (highly susceptible): black lesions covering 76-100% of the SCI.

1000-kernel weight (TKW) and yield estimation

Three central rows of each replicate plot were harvested at maturity stage to measure grain yield (gr/plant). A 500-seed subsample from each row was used to calculate 1000-kernel weight (TKW). The number of tillers per plant was determined on individual hand-harvested plants.

Statistical analysis

Data was subjected to analysis of variance using the STAT-ITCF statistical programme (2nd Version). Differences between means were evaluated for significance by using Newman-Keuls test at 5% probability level (Anonymous, 1988)

Results and Discussion

CRR produced brown-dark lesions on SCIs, and these symptoms were more severe on the susceptible cultivar Bouhouth7 (Fig. 1). The results are in agreement with our pre-

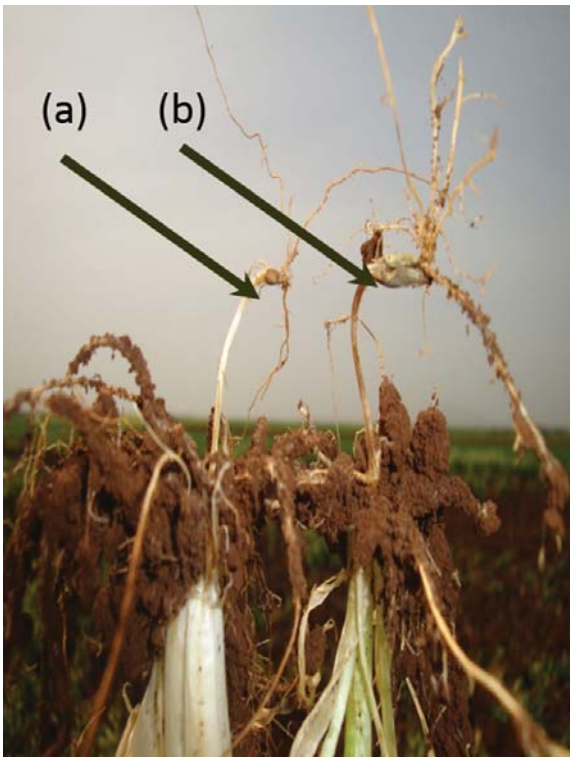

Figure 1. Common root rot symptoms (*Cochliobolus sativus*)on the wheat (a) highly tolerant landrace 'Horani' and(b) highly susceptible cv. Bouhouth 7, under field conditions.

vious observations under natural field conditions (Arabi and Jawhar, 2002). The reactions of the 10 wheat cultivars to *C. sativus* are presented in Table 1. Significant differences ($P<0.05$) in disease severity were detected among cultivars, with values being consistently higher in the susceptible cultivars, in both years of experimentation. In both seasons, landrace Salamoni was highly susceptible with mean disease severity 83.4 %. The *T. durum* landrace Horani proved to be the most tolerant having 9.9% disease severity (Table 1). In general, the *T. durum* cultivars were more tolerant than those of *T. aestivum* (Table 1).

The effects of CRR on grain yield are presented in Table 2. During the first growing season (2016), no significant differences in yield were observed between plants obtained from inoculated and non-inoculated seeds. During the second growing season (2017), grain yield was reduced by CRR in relation to the non-inoculated seeds in all other cultivars except for the highly tolerant landrace Horani.

Moreover, CRR significantly ($P<0.05$) reduced the TKW of the cvs Bouhouth6 and Maksibak by 18.9 % and 8.6 % in 2016, and by 14.3% and 29.5 % in 2017, respectively (Table 3). The reduction of TKW in the other cultivars differed greatly depending on the cultivar (Table 3).

As shown in Table 4, the number of tillers decreased significantly ($P<0.05$) by 28 and 27% in the cvs Bouhouth6 and Cham3, in 2016, and by 37.5 and 39.5%, in 2017, respectively (Table 4). Diseased plants had fewer tillers resulting in reduced grain yield per plant. Similar results were reported by Fernandez *et al.* (2014) and Duczek and Jones-Flory (1993), who found that wheat plants infected by *C. sativus* early in the season produced fewer tillers than those infected later in the season, which was reflected in yield per plant. The current study also showed that the average response of wheat cultivars to CRR differed with the susceptibility level. These findings are in agreement with those of Rush and Mathieson (1990) and Bhandari and Shrestha (2004).

Overall, CRR had a negative effect on TKW and the number of tillers produced in susceptible wheat cultivars grown under rainfed conditions in southern Syria. The reduction in total grain yield may be attributed mainly to the reduction in the number of tillers, as reported by Conner *et al.* (1996). However, according to Fernandez and Con-

Table 1. Reaction of wheat genotypes to Common root rot (CRR; *Cochliobolus sativus*) under field conditions in two growing seasons (2016, 2017).

Cultivar	Origin	Severity (% subcrown internodes infected area)		
		Year 2016	Year 2017	Mean effect
Horani	Landrace	A11.3d[y]	A8.5d	9.9d
Cham3	Syrian (Developed by SGCASR)*	A10.3d	A9.2d	9.7d
Doma4	"	A11.2d	B15.9d	13.6d
Cham2	"	A15.2d	B22.5d	18.9c
Doma1	"	A31.2c	B17.0d	24.1c
Bouhouth4	"	A33.2c	B27.9c	30.6c
Bouhouth6	"	A42.6ab	B48.2ab	45.4ab
Maksibak	Introduced	A66.5ab	B58.9b	62.7b
Bouhouth7	Syrian (Developed by SGCASR)*	A84.9a	B77.2a	81.1a
Salamoni	Landrace	A82.97a	A84.0a	83.5a
Mean		A42.11	B36.92	

[y] Means (three replicates/cultivar) preceded by different capital letters (row) and followed by different lowercase letters (column) differ significantly at $P<0.05$ according to Newman-Keuls test. *SGCASR: Syrian General Commission of the Agricultural Scientific Research.

Table 2. Effect of Common root rot (CRR; *Cochliobolus sativus*) on grain yield in wheat cultivars under field conditions in two growing seasons (2016, 2017).

Cultivar	Grain yield (g/plant)			
	Year 2016		Year 2017	
	Non	Ino.	Non	Ino.
Horani	A2.4c[y]	A2.0c	A18.2bc	A16.7ab
Cham3	A4.2bc	A4.0bc	A16.7C	B7.7c
Doma4	A3.6c	A4.6bc	A33.0a	B19.1a
Cham2	A8.2ab	A8.9a	A18.7bc	B8.7c
Doma1	A4.5bc	A4.7bc	A16.7bc	B13.6bc
Bouhouth4	A2.9c	A3.0c	A19.8bc	B12.3bc
Bouhouth6	A5.8bc	A3.8bc	A28.3ab	B11.5bc
Maksibak	A2.8c	A3.2c	A16.9bc	B9.9c
Bouhouth7	A4.8bc	A4.1bc	A18.2bc	B12.6bc
Salamoni	A11.1a	A7.1ab	A25.3abc	B10.4c
Mean	A5.03	A4.5	A20.9	B12.9
Mean	B4.8		A16.6	

[y] Means (three replicates/cultivar) preceded by different capital letters (row) and followed by different lowercase letters (column) differ significantly at $P<0.05$ according to Newman-Keuls test. Non: Non-inoculated seeds (control), Ino.: Inoculated seeds (Kokko *et al*., 1995).

Table 3. Effect of Common root rot (CRR; *Cochliobolus sativus*) on 1000-kernel weight (TKW) of wheat cultivars during two growing seasons (2016, 2017).

Cultivar	1000-kernel weight (g)			
	Year 2016		Year 2017	
	Non	Ino.	Non	Ino.
Horani	A36.0ay	B34.0b	A37.6ab	B34.0b
Cham3	A28.6bc	A27.3bc	A35.3ab	B27.3bc
Doma4	A34.0ab	A33.0bc	A37.0ab	B30.6bc
Cham2	A28.1bc	A29.0bc	A28.6ab	A28.6bc
Doma1	B39.0a	A40.6a	A41.0a	A38.6a
Bouhouth4	B23.0c	A24.6c	A29.6b	B24.6c
Bouhouth6	A37.0a	B30.0bc	A35.0ab	B30.0bc
Maksibak	A28.0bc	B25.6bc	A36.3ab	B25.6bc
Bouhouth7	B25.6c	A28.3bc	A32.3ab	B28.3bc
Salamoni	B28.6bc	A27.6bc	A35.3ab	B32.6bc
Mean	A30.8	B28.0	A35.5	B30.1
Mean	B29.7		A32.8	

[y] Means(three replicates/cultivar) preceded by different capital letters (row) and followed by different lowercase letters (column) differ significantly at $P<0.05$ according to Newman-Keuls test. Non: Non- inoculated seeds (control), Ino.: Inoculated seeds (Kokko *et al*., 1995).

ner (2011), CRR directly affected the carbon fixation and other physiological processes in wheat leaves by reducing the upward movement of water and nutrients in plants.

CRR had a direct impact on total grain yield of wheat, and therefore, this disease should be considered when managing wheat diseases. Moreover, continued ef-

Table 4. Effect of Common root rot (CRR; *Cochliobolus sativus*) on the number of tillers of wheat cultivars during two growing seasons (2016, 2017).

Cultivar	Number of tillers/plant			
	Year 2016		Year 2017	
	Non	Ino.	Non	Ino.
Horani	A5.6by	A5.6a	A6.0a	B5.3a
Cham3	A6.3ab	B4.6ab	A7.6a	B4.6a
Doma4	A8.0b	B6.3a	A7.0a	B6.3a
Cham2	A5.3b	B4.6ab	A6.6a	B5.0a
Doma1	A5.6b	B5.0ab	A6.6a	B5.6a
Bouhouth4	A6.3ab	B5.6a	A6.3a	B5.3a
Bouhouth6	A5.0b	B3.6b	A8.0a	B5.0a
Maksibak	A5.0b	A5.0ab	A6.6a	B5.0a
Bouhouth7	A6.3ab	B5.6a	A6.3a	B5.0a
Salamoni	A7.6a	B6.0a	A7.6a	B4.3a
Mean	A6.1	A5.2	A6.9a	B5.1a
Mean	A5.2		A5.1	

y Means (three replicates/cultivar) preceded by different capital letters (row) and followed by different lowercase letters (column) differ significantly at P<0.05 according to Newman-Keuls test. Non: Non-inoculated seeds (control), Ino.: Inoculated seeds (Kokko *et al*., 1995).

forts are required to monitor the occurrence of CRR in cereal fields in Syria to develop a better understanding of the potential risk of its establishment and intensification. The highly CRR tolerant landrace Horani can be considered as a promising parent in wheat breeding programmes.

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Απόκριση παλαιών, νέων και γηγενών Συριακών γονοτύπων σίτου στην ασθένεια "κοινή σήψη ριζών" σε συνθήκες αγρού

M.I.E. Arabi, E. Al-Shehadah και M. Jawhar

Περίληψη Η απόκριση ευρέως καλλιεργούμενων και γηγενών Συριακών ποικιλιών σίτου στη μόλυνση από το μύκητα *Cochliobolus sativus*, αξιολογήθηκε μετά από σύγκριση πειραματικών τεμαχίων με και χωρίς τεχνητή μόλυνση κατά τη διάρκεια δύο διαδοχικών καλλιεργητικών περιόδων. Τα αποτελέσματα έδειξαν ότι η απόκριση στο παθογόνο διέφερε ανάλογα με το επίπεδο ευπάθειας των ποικιλιών σίτου και ότι η ασθένεια μείωσε σημαντικά (P <0,05) την παραγωγή, το βαθμό αδελφώματος και το βάρος των σπόρων Τα προσβεβλημένα φυτά εμφάνιζαν μικρότερο βαθμό αδελφώματος με αποτέλεσμα τη μείωση της παραγωγής ανά φυτό. Η απώλεια στην παραγωγή των ποικιλιών του *Triticum durum* ήταν μεγαλύτερη από αυτή των ποικιλιών του *Triticum aestivum*. Επιπλέον, η γηγενής ποικιλία Horani του *T. durum* εμφάνισε το υψηλότερο επίπεδο αντοχής στην ασθένεια. Ως εκ τούτου, η συγκεκριμένη ποικιλία θα μπορούσε να είναι υποψήφιος δότης ανθεκτικότητας στην ασθένεια σε μελλοντικά προγράμματα βελτίωσης ποικιλιών. Επειδή κάτω από ευνοϊκές συνθήκες η ασθένεια μπορεί να προκαλέσει σημαντική μείωση της παραγωγής σίτου, συνιστάται η εφαρμογή μέτρων διαχείρισης που θα μειώσουν την ένταση της προβολής.

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Plant parasitic nematodes fauna in citrus orchards in Khuzestan province, Southwestern Iran

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Summary During a survey on the biodiversity of plant-parasitic nematodes in citrus orchards of Khuzestan province (Southwestern Iran), 97 root and soil samples were collected. Nematodes were extracted and identified using morphological and morphometric diagnostic characters. Six nematode species were identified, namely: *Helicotylenchus abunaamai, H. crenacauda, Pratylenchus allius, P. musii, Psilenchus hilarulus* and *Tylenchulus semipenetrans*. Except *T. semipenetrans*, the remaining five species were found only in the rhizosphere of citrus, not in citrus roots, and their pathogenicity on citrus plants was not further studied. This is the first record of *P. allius* and *P. musii* for the nematode fauna in Iran. *H. crenacauda* is a new record for the nematode fauna in the Khuzestan province and is reported for the first time in citrus orchards in Iran. To our knowledge, this is the first report of *H. abunaamai* in citrus orchards worldwide.

Additional keywords: citrus, first record, morphology, morphometric, plant-parasitic

Introduction

Citrus is indigenous to southeastern Asia but has existed in Mediterranean basin for centuries. Species of citrus have great importance in some Mediterranean regions (Duarte *et al.,* 2016). Iran is the eighth largest producer of citrus in the world. In 2017, Iranian citrus fruit production reached 4,067,000 tons (FAOSTAT, 2017). Khuzestan province is one of the major citrus-producing regions in the country.

A wide range of plant-parasitic nematodes has been associated with the citrus rhizosphere but only some species cause damage to the trees (Verdejo-Lucas and McKenry, 2004). The citrus nematode (*Tylenchulus semipenetrans* Cobb, 1913) causes a slow decline of citrus all around the world and restricts citrus fruit production under a wide spectrum of environmental conditions (Duncan, 2005). Spreading decline is a serious disease of citrus caused by *Radopholus similis* (Cobb, 1893) Thorne, 1949 that only occurs in Florida's central ridge (Duncan, 2005). *R. citri* Machon and Bridge (1996) was found in citrus roots in Indonesia and was associated with very severe necrosis and root destruction (Machon and Bridge, 1996).

Pratylenchus coffeae (Zimmermann, 1898) Filipjev and Schuurmans Stekhoven, 1941, *P. brachyurus* (Godfrey, 1929) Filipjev and Schuurmans Stekhoven, 1941 and *P. vulnus* Allen and Jensen (1951) are three species of lesion nematodes associated with the citrus tree. Also, *Belonolaimus longicaudatus* Rau (1958) causes damage to citrus. Root-knot nematodes (*Meloidogyne* spp.) are able to attack citrus and are confined to prevent dissemination. Pathogenic species of root-knot nematode were reported from Taiwan and New Delhi (Duncan, 2005).

Many populations of *Xiphinema brevicollum* Lordello and Da Costa (1961) have been associated with the decline of grapefruit trees in Sudan (Yassin, 1974). *Paratrichodorus lobatus* (Colbran, 1965) has also been found in high numbers in citrus nurseries in Australia (Stirling, 1976). *Hemicycliophora arenaria* (Raski, 1958) is a species native to plants in the southern California that causes damage in citrus nurseries (McElroy *et al.,* 1966). *Caloosia nudata* (Colbran) Brzeski, 1974 causes similar symptoms on citrus in Australia (Colbran, 1963).

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A plentiful of plant-parasitic nematode species have been associated with the citrus rhizosphere in Iran. *T. semipenetrans* and *Diphtherophora communis* (de Man, 1880) have been associated with citrus in Fars province (Abivardi *et al.,* 1970). *Meloidogyne javanica* (Treub, 1885) Chitwood, 1949 from sour orange (*C. aurantium*) in Khuzestan province (Akhiani *et al.,* 1984), *Hemicriconemoides chiwoodi* Esser (1960), *Helicotylenchus pseudorobustus* (Steiner, 1914) Golden, 1956, *Boleodorus thylactus* Thorne (1941) from orange (*C. sinensis*) in Kerman province (Jahanshahi afshar *et al.,* 2006), *Scutellonema brachyurus* (Steiner, 1938) Andrassy, 1958 from citrus in Golestan province (Tanha Maafi *et al.,* 2006) and *Helicotylenchus macronatus* Mulk and Jairajpuri (1975) have been reported from lemon (*C. limon*) in Kerman province (Ali Ramaji *et al.,* 2006).

In the study by Divsalar *et al.* (2011), 27 species of plant-parasitic nematodes have been identified from the citrus rhizosphere in Gilan and Mazandaran as *Criconemoides xenoplax* Raski (1952), *Filenchus facultativus* (Szczygiel, 1970) Raski and Geraert (1987), *Helicotylenchus exallus* Sher (1966), *H. vulgaris* (Yuen, 1964), *Ogma civellae* (Steiner, 1949) Raski and Luc (1987), *Paratylenchus nanus* (Coob, 1923), *Pratylenchus loosi* (Loof, 1960), *P. neglectus*, *P. jaehni*, *P. zea* and *Psilenchus hilarulus* (de Man, 1921). Also, *Hemicriconemoides chitwoodi* and *Tylenchorhynchus agri* (Ferris, 1963) have been found associated with the rhizosphere of citrus in Kerman province (Rashidifard *et al.,* 2014).

There is very little information about plant-parasitic nematodes associated with citrus orchards in Khuzestan province. To fill this gap, this study aimed to determine the plant parasitic nematodes of citrus in Khuzestan province, Southwestern Iran using morphological and morphometric data.

Material and methods

About 97 root and soil samples were collected from citrus orchards in the Khuzestan province, Southwestern Iran. *T. semipenetrans* was extracted from the roots based on the Coolen and D'Herde method (1972). Roots were washed, cut in pieces and processed for nematode extraction by blender followed by centrifugal flotation. The roots were stained (Hooper *et al.,* 2005) and immature and mature females were observed on the root surfaces.

Soil samples were taken from 5 to 40 cm depth from different regions. Then the soil samples were put in a polyethylene bags with pertinent information about each sample, then brought to the laboratory and kept in the refrigerator at about 4°C until they were processed for nematode extraction.

The Jenkins (1964) method was used to extract the nematodes from soil samples. The collected specimens were killed in hot 4% formaldehyde solution, transferred to anhydrous glycerin according to De Grisse's (1969) method. In some samples, the tray method (Whitehead and Hemming, 1965) was employed to obtain a suspension of nematodes from the soil. Nematodes were mounted in a small drop of glycerin on permanent slides. Observations and measurements were done using an Olympus CX31 light microscope equipped with a drawing tube. Some of the best-preserved specimens were photographed using an Olympus DP12 digital camera attached to an Olympus BX51 light microscope. Nematode species were identified based on morphological and morphometric characters using valid keys such as Siddiqi 2000; Castillo and Vovlas, 2007; Geraert, 2008; Geraert, 2013.

Results and discussion

Based on morphological and morphometric characters, six species of plant-parasitic nematodes were identified, namely: *Helicotylenchus abunaamai* Siddiqi (1972), *H. crenacauda* Sher (1966), *Pratylenchus allius* (Shahina and Maqbool, 1996) Siddiqi (2000), *P. musii* Choudhury and Phukan (1989), *Psilenchus hilarulus* de Man (1921) and *Tylenchulus semipenetrans* Cobb (1913). Except *T. semipenetrans*, the remaining five species were found only in the rhizo-

sphere of citrus, not in citrus roots. No further studies were performed on pathogenicity of these species on citrus plants.

Morphometric measurements of the identified nematodes closely corresponded with the published reports; nevertheless, insignificant morphological and morphometric differences were observed in some species are discussed below. The most important morphological characters of the considered species are illustrated in Figures 1-6. The morphometrics of the considered species are given in Tables 1-4.

Helicotylenchus abunaamai (Siddiqi, 1972)

Figure 1 (a-h)

MEASUREMENTS (Table 1)

The general morphology of the recovered population of the species resembles the characters given in the original description (Siddiqi, 1972). However, the length of the stylet is slightly shorter (18-21.5 *vs.* 21-22 µm). This species has been reported from Malaysia (Sauer and Winoto, 1975), Pakistan (Firoza and Maqbool, 1991), Thailand (Mizukubo *et al.,* 1992) and Turkey (Kepenekci, 2002). There is no significant difference between our population and these populations.

Kashi and Karegar (2014) reported on the presence of the same species from sugarcane in Haft-Tappeh, Khuzestan province, Southwestern Iran. Their population has differences with the main description and our population. These differences are in body length (600-779 *vs* 515-611 µm), ratio c (24.2-33.7 *vs* 37.6-48.2), ratio c' (1.4-1.98 *vs* 1-1.2), length of stylet (23.3-26.8 *vs* 18-21.6 µm) and tail length (20-29 *vs* 11-14 µm).

In the present study, this species was recovered from 8.8% of soil samples from the rhizosphere of citrus, sour orange, lemon and tangerine in the vicinity of Dezful city, Khuzestan province, Southwestern Iran. To our knowledge, this is the first report of *H. abunaamai* in citrus orchards worldwide.

Helicotylenchus crenacauda (Sher, 1966)

Figure 1 (i-p)

MEASUREMENTS (Table 1)

The general morphology of the recovered population of the species resembles the characters given in original description (Sher, 1966). This species has been reported from the rhizosphere of rice in Gilan (Pedramfar *et al.,* 2002 and Kashi and Karegar, 2014), ornamental plants in Mahallat (Mohammad Deimi *et al.,* 2008) and vineyards in Markazi province, Iran (Mohammad Deimi and Mitkowski, 2010).

The population of Khuzestan province did not differ significantly from the populations of Gilan. Compared to the Mahallat population, the ratios a, b' and c are lower (25.4-29.7 *vs* 31.2-37.4, 4-4.9 *vs* 5.1-6.5 and 35-45.2 *vs* 47.9-57.8 respectively). Also, stylet length is shorter (24-25 *vs* 25-29 µm). Compared to Markazi province, the ratio c is lower (35-45.2 *vs* 45.2-53.5).

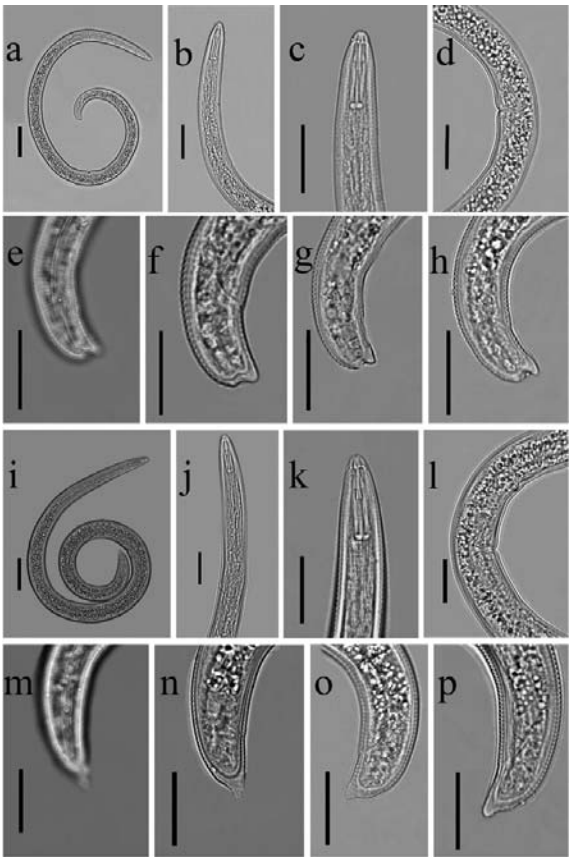

Figure 1. a-h: *Helicotylenchus abunaamai*. a: Entire body; b, c: Anterior region; d: Vulval region; e: Tail showing phasmid; f-h: Variations of tail shape. i-p: *Helicotylenchus crenacauda*. i: Entire body; j, k: Anterior region; l: Vulval region; m: Tail showing phasmid; n-p: Variations of tail shape. (Scale bars: a, i = 50 µm, b-h, j-p = 20 µm).

Table 1. Morphometrics of *Helicotylenchus abunaamai* and *Helicotylenchus crenacauda* recovered from citrus orchards of Khuzestan province, southwestern Iran. All measurements are in μm and in the form: mean ± s.d. (range).

Character	*Helicotylenchus abunaamai*	*Helicotylenchus crenacauda*
n	10	12
L	560.6 ± 31.5 (515-611)	628.5 ± 41.3 (563-698)
a	28.7 ± 2.6 (25.1-32)	27 ± 1.5 (25.4-29.7)
b	5.3 ± 0.4 (4.6-5.8)	5.2 ± 0.4 (4.5-6.1)
b′	4.4 ± 0.3 (3.9-4.9)	4.4 ± 0.2 (4-4.9)
c	44.6 ± 3.3 (37.6-48.2)	40 ± 3.5 (35-45.2)
c′	1.1 ± 0.1 (1-1.2)	1.2 ± 0.1 (1-1.4)
V	63.8 ± 1.1 (62.6-66.5)	62.2 ± 1 (61-63.8)
Lip height	3.7 ± 0.4 (3-4.5)	3.9 ± 0.3 (3.5-4)
Lip width	5.4 ± 0.4 (4.5-6)	5.8 ± 0.5 (5-6.5)
Stylet length	20.5 ± 1.2 (18- 21.5)	24.4 ± 0.5 (24-25)
Conus length	9.8 ± 1 (8.5-11.5)	11 ± 0.7 (10-12)
DGO	9.2 ± 0.9 (8.5-10)	10.2 ± 1.2 (9-12)
Pharynx length	106.5 ± 7.9 (93.5-117)	116.7 ± 4.2 (111-122.5)
Pharyngeal glands	127.2 ± 8.2 (117-138)	140.3 ± 4.7 (134-148)
Excretory pore	90 ± 6.3 (80-102)	103.5 ± 4.4 (99-112)
Median bulb	64.7 ± 2.8 (59.5-69)	74.7 ± 2.7 (72-78)
Body width	19.5 ± 1.3 (17.5-21.5)	23.2 ± 2 (19-25)
Tail length	12.4 ± 0.8 (11-14)	16 ± 1.5 (14-18)
Anal body width	11.5 ± 0.5 (11-12)	12.8 ± 0.8 (11.5-14)
Vulva body width	19.5 ± 1.3 (17.5-21.5)	23.2 ± 2 (19-25)
Vulva-Anus	195 ± 16.4 (170-216)	222.6 ± 13.1 (204-237)
Phasmids from tail terminus	15.6 ± 0.6 (13.5-18)	21 ± 1.6 (18-23)

In the present study, this species was recovered from 10.7% of soil samples from the rhizosphere of sour orange and lemon in the vicinity of Ahvaz (GPS coordinates: 31° 19′ 5.9″ N 48° 40′ 14.2″ E), Dezful, Izeh (GPS coordinates: 31° 49′ 26.3′′ N, 49° 52′ 12.3′′ E) and Baghmalek cities, Khuzestan province, Southwestern Iran. This is the first report of *H. crenacauda* in citrus orchards in Iran and a new record for the nematodes fauna in Khuzestan province.

Pratylenchus allius
(Shahina and Maqbool, 1996)
Siddiqi (2000)
Figures 2, 3 (a-d)
MEASUREMENTS (Table 2)
DESCRIPTION

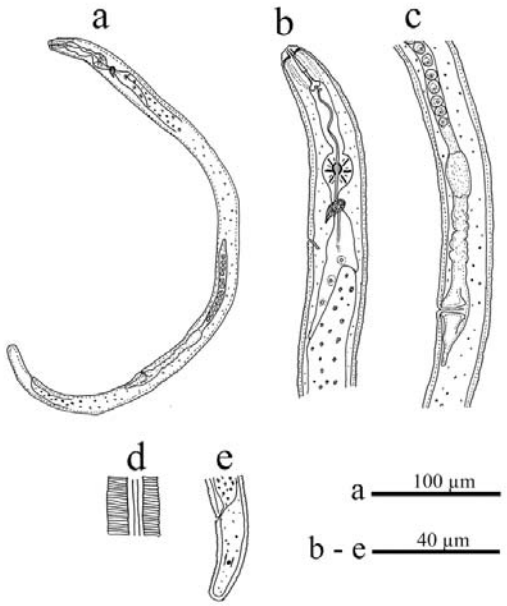

Figure 2. Female of *Pratylenchus allius*. a: Entire body; b: Anterior region; c: Vulval region; d: Lateral field at mid-body; e: Tail.

Female: Nematodes are of small size (410-490 µm long), with body strongly arcuate upon fixation. Cuticular annulation distinct, 0.8-1.2 µm wide at mid-body. Lateral field with four incisures, not areolated, occupying about one-third of body diameter. The labial region is low, flattened and not off set, 2.1-3.5 µm high and 6-7.2 µm wide at base, with three annuli. The framework is strongly sclerotized. The stylet is strong and relatively short, Stylet knobs well-developed, rounded, 1.8-2.4 µm high and 3-4 µm wide. The median bulb is oval in shape, very muscular, 11.8-12.6 µm long and 8.4-8.6 µm wide. Nerve ring surrounds isthmus. Oesophageal glands overlap the intestine ventrally about two times the body width, three gland nuclei in tandem. Excretory pore almost at the level of the pharyngo-intestinal valve. Hemizonid is about 2-3 annuli wide, 1-3 annuli anterior to excretory pore. Genital system monodelphic-prodelphic, ovary outstretched. Anterior branch is well developed, 130-140 µm long. Spermatheca oval, without sperm. Ovary with oocytes arranged in one or two rows. Vulva a transverse slit, posteriorly located, lateral flaps and epiptygma absent. The vagina is about 7-9 µm long. Post-vulval uterine sac about one-time vulval body width. Tail cylindrical, terminus rounded, without annulation. Phasmids pore-like in shape, 10-15 annuli anterior from the tail terminus.

Male: Not found.

REMARKS

The Iranian population of *P. allius* is very similar to the type population of the species from Azad Kashmir, Pakistan (Shahina and Maqbool, 1996). However, the length of DGO and the number of tail annuli in our population are slightly higher than that given in the original description (0.9-1.3 *vs* 0.6-1 µm and 22-33 *vs* 25-26 respectively).

This species was originally described by Shahina and Maqbool (1996) as *Radopholus allius*. Siddiqi (2000) examined the paratype of the species and found that it belongs to the genus *Pratylenchus*. Castillo and Vovlas (2007) considered *P. allius* as a new junior synonym of *P. thornei*. Geraert (2013) considered *P. allius* as a separate species from *P. thornei* and mentioned that the stylet is larger in *P. thornei* (15-19 *vs* 14-15.5 µm). We found more differences. In *P. allius*, the length of DGO is shorter (0.6-1 *vs* 2-3 µm), without male (*vs* rare male), tail tip only rounded (*vs* bluntly rounded or truncate) in *P. thornei*. We agree with Geraert's opinion and *P. allius* is considered here as separate species from *P. thornei*.

In the present study, this species was recovered from 14.7% of soil samples from the rhizosphere of orange and tangerine in the vicinity of Shush and Baghmalek cities, Khuzestan province, Southwestern Iran. This is the first record of *P. allius* for the nematode fauna in Iran.

Pratylenchus musii
Choudhury and Phukan (1989)

Figures 3 (e-m), 4

MEASUREMENTS (Table 2)

DESCRIPTION

Female: Nematodes are of small size (386-450 µm long), with the body slightly ventrally arcuate upon fixation. Cuticular annulation relatively fine, 0.8-1.2 µm wide at mid-body. Lateral field with six incisures, not areolated, occupying about one-third

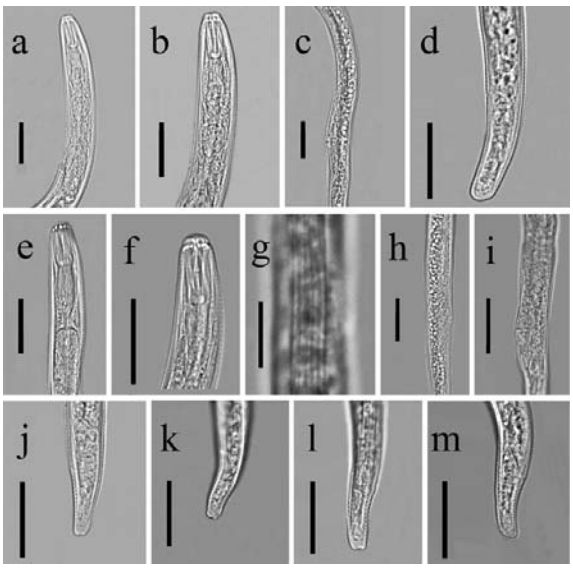

Figure 3. a-d: Female of *Pratylenchus allius*. a, b: Anterior region; c: Vulval region; d: Tail. e-m: Female of *Pratylenchus musii*. e, f: Anterior region; g: Lateral field at mid-body; h, i: Vulval region; j-m: Variations of tail shape. (Scale bars: 20 µm).

Table 2. Morphometrics of *Pratylenchus allius* and *Pratylenchus musii* recovered from citrus orchards of Khuzestan province, southwestern Iran. All measurements are in *µ*m and in the form: mean ± s.d. (range).

Character	*Pratylenchus allius* Khuzestan province	*Pratylenchus allius* (Shahina and Maqbool 1996) Siddiqi 2000	*Pratylenchus mussi* Khuzestan province	*Pratylenchus mussi* Geraert (2013)
n	15	10	10	-
L	461.8 ± 23.4 (410-490)	420-480.5	418 ± 21.7 (386-450)	430-490
a	31.1 ± 2.5 (27-36.5)	27.5-30	27.9 ± 2.5 (25.2-32.1)	30-34
b	5.8 ± 0.8 (4.4-7.2)	6.4-6.7	4.9 ± 0.5 (4-5.6)	-
b′	4.4 ± 0.6 (3.4-5.8)	4.5-4.6	3.8 ± 0.4 (3.2-4.8)	-
c	20.6 ± 2.3 (17.3-25)	17-20	22.3 ± 2 (19.2-24.6)	18-23
c′	2.3 ± 0.5 (2-2.8)	2.3-2.7	2.3 ± 0.3 (1.8-2.7)	1.7-2.5
V	76 ± 1.7 (73-79.2)	76-77	82.6 ± 1.2 (80-84.2)	78-83
DGO	1 ± 0.1 (0.9-1.3)	0.6-1	3.5 ± 0.8 (2.5-5)	3-4
Stylet length	14.7 ± 0.7 (14-15.5)	14-15.5	14.7 ± 0.8 (13.5-15.5)	14-15
Stylet shaft	7.4 ± 0.6 (6.5-8.5)	-	8.2 ± 0.5 (8-9)	-
Median bulb	46.2 ± 4.6 (41-57.5)	40-43	43.8 ± 3.2 (40-51)	-
Excretory pore	65.5 ± 6.9 (50-74.5)	60-68	67.6 ± 6 (57-78.5)	68-77
Pharynx length	80.7 ± 10.4 (64-103)	64-68	83.5 ± 6.7 (75-93)	98-112
Pharyngeal overlap	104.3 ± 11.1 (84-123)	99-103	109.4 ± 7 (99-120)	-
head-nerve ring	57.6 ± 4.2 (54-67)	-	56.8 ± 4.4 (52-66.5)	-
Body width	14.8 ± 1.4 (12.5-17.5)	16	15 ± 0.7 (14-15.5)	14
Anal body width	9.1 ± 0.5 (8.5-10)	-	7.9 ± 0.8 (6.5-9)	-
Vulval body width	14.6 ± 1.3 (12.5-17.5)	-	12.8 ± 0.4 (12.5-14)	-
V-anus	90.5 ± 10 (76-110.5)	-	52.6 ± 4.6 (47.5-60)	-
PVUS	14.9 ± 1 (13-17)	-	14.3 ± 1.6 (14-18.5)	22-30
Tail length	22.6 ± 2.5 (19-27)	24-26.4	18.8 ± 1.6 (17-21.5)	19-23
Lateral field width	4.9 ± 0.5 (4-6)	-	4.4 ± 0.5 (3.5-5)	-
Phasmids from tail terminus	12.9 ± 1.2 (11-15)	-	12.4 ± 1.1 (11-14.5)	10-16
Num. of tail annuli	28.8 ± 3.2 (22-33)	-	20 ± 1.7 (18-23)	20-26

of body diameter. The labial region is low, flattened and slightly off set, 1.6-2.3 µm high and 6-7.8 µm wide at base, with two annuli. The framework is strongly sclerotized. The stylet is strong and relatively short, Stylet knobs well-developed, rounded, 1.8-2.4 µm high and 3.6-4.5 µm wide. The median bulb is oval in shape, 10.8-12 µm long and 9.6-11.4 µm wide, 40-51 µm from anterior end. Nerve ring surrounds isthmus. Oesophageal glands overlap the intestine ventrally about two times the body width, three gland nuclei almost in tandem. Excretory pore slightly higher than the pharyngo-intestinal valve. Hemizonid is about two annuli wide, 1-3 annuli anterior to excretory pore. Genital system monodelphic-prodelphic, ovary outstretched. Anterior branch is well developed, 132-143 µm long. Spermatheca is oval in shape, with sperm. Ovary with oocytes arranged in one or two rows. Vulva a transverse slit, posteriorly located, lateral flaps and epiptygma absent. The vagina is about 6.5-8 µm long. Post-vulval uterine sac more than one-time vulval body width. Tail cylindrical, terminus rounded, with annulation. Phasmids pore-like in shape, 11-15 annuli anterior from the tail terminus.

Male: Not found.

REMARKS

According to the morphological characters and morphometric data given in Geraert (2013), there were no differences between the Iranian population of *P. musii* and the original description. However, the post-uterine sac length is shorter (14-18.5 *vs* 22-30 µm).

The species was originally recovered from the rhizosphere of banana and described by Choudhury and Phukan, 1989 from Assam, India (Geraert, 2013). In a study of nematode community associated with banana in Assam, India, Deori *et al.,* 2014 found that *P. musii* was one of the predominant nematode species around the banana rhizosphere.

In the present study, this species was recovered from 9.8% of soil samples from the rhizosphere of orange and tangerine in the vicinity of Shush city, Khuzestan province, Southwestern Iran. This is the first record of *P. musii* for the nematode fauna in Iran.

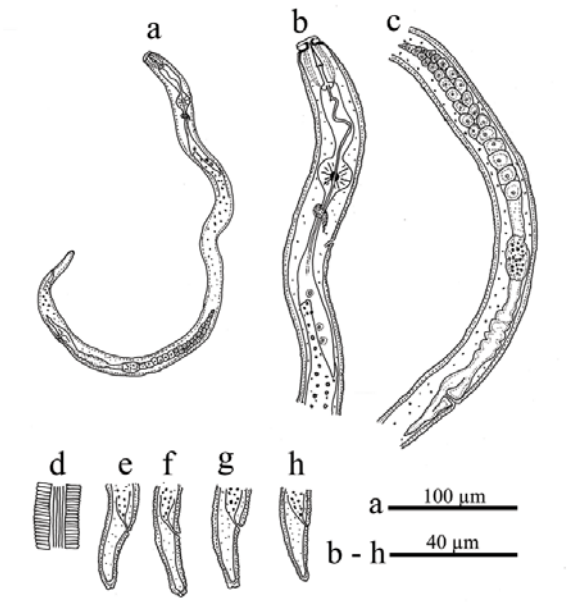

Figure 4. Female of *Pratylenchus musii*. a: Entire body; b: Anterior region; c: Vulval region; d: Lateral field at mid-body; e-h: Tail.

Psilenchus hilarulus de Man (1921)

Figure 5

MEASUREMENTS (Table 3)

Iranian population of *P. hilarulus* is in morphological and morphometric agreement with the original description (Geraert, 2008). However, the length of female body is shorter (640-845 *vs* 890-1150 µm). This species has been reported from the citrus rhizosphere in Mazandaran province, Iran (Divsalar *et al.,* 2011). Also, has been reported from the rhizosphere of sugarcane in Khuzestan province, Iran (Kheiri, 1995). In the present study, this species was recovered from 20.5% of soil samples from the rhizosphere of orange, lemon and sour orange in the vicinity of Shush, Dezful, Ramhormoz, Baghmalek and Ramin cities, Khuzestan province, Southwestern Iran.

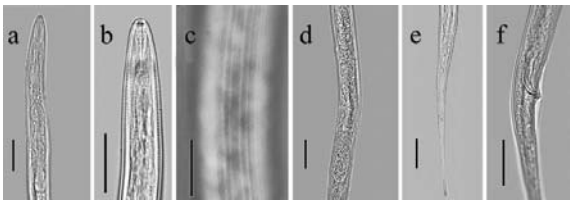

Figure 5. *Psilenchus hilarulus*. a, b: Anterior region; c: Lateral field at mid-body; d: Vulval region; e: Female tail; f: Male tail. (Scale bars: 20 µm).

Tylenchulus semipenetrans Cobb (1913)

Figure 6

MEASUREMENTS (Table 4)

Characters measured in Khuzestan population of *T. semipenetrans* are consistent

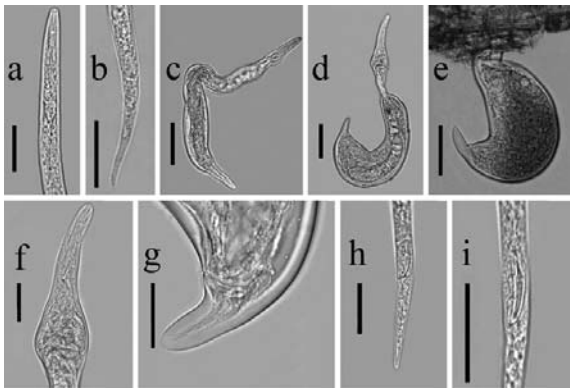

Figure 6. *Tylenchulus semipenetrans*. a: Anterior region of Juvenile; b: Posterior region of Juvenile; c: Immature female; d, e: Mature female; f: Anterior region of female; g: Posterior region of female; h, i: Male tail. (Scale bars: a, b = 20 µm, c-e = 50 µm, f-i = 20 µm).

Table 3. Morphometrics of *Psilenchus hilarulus* recovered from citrus orchards of Khuzestan province, southwestern Iran. All measurements are in *μ*m and in the form: mean ± s.d. (range).

Character	*Psilenchus hilarulus*	
	Female	Male
n	11	12
L	760 ± 82 (640-845)	737.4 ± 78.3 (643- 841)
a	46.5 ± 2.1 (43.3-48.9)	45.3 ± 5 (36.7-52.9)
b	6 ± 0.6 (5-6.7)	5.9 ± 0.7 (4.8-6.8)
c	7 ± 0.5 (6-7.6)	6.1 ± 0.4 (5.6-6.7)
c′	10.1 ± 0.9 (8.6-11.6)	10.1 ± 1 (8.5-12.2)
V	48.8 ± 1.9 (44.5-51.3)	-
V′	57.1 ± 1.8 (54.7-59.3)	-
Stylet length	13.3 ± 0.7 (12-14.5)	13.1 ± 0.3 (12.5-14)
m	35.9 ± 5 (28.5-45.5)	36.9 ± 3.3 (32-43)
DGO	4.5 ± 0.8 (3.5-6)	4.3 ± 0.7 (3.5-6)
Oesophagus	129 ± 4.9 (125-140)	125 ± 8 (108.5-140)
MB	54.7 ± 1.5 (52-57.5)	54.7 ± 1.9 (52-59)
Body width	16.8 ± 1.7 (14-19)	16.4 ± 1.9 (13-18.5)
Excretory pore	93.4 ± 5.9 (84-105)	92 ± 7.1 (86-105.5)
Vulval body width	14.8 ± 1.3 (13.5-16)	-
Vulva-anus	285.8 ± 40 (218-345)	-
Anal body width	10.2 ± 0.7 (9.5-11)	11.3 ± 0.9 (11-12.5)
Tail length	117 ± 8.5 (108-138)	120 ± 7.6 (112-132)
T/VA	0.4 ± 0 (0.34-0.48)	-
Spicule length	-	22.1 ± 2.1 (20-24.5)
Gubernaculum length	-	7.5 ± 0.6 (6.5-8.5)
Bursa	-	43.9 ± 8.7 (30-57)

with other populations including Inserra *et al.* (1988) and Rashidifard *et al.* (2015). However, the length of the stylet in second stage juveniles (J2) is slightly shorter (9.5-12.5 *vs* 12.2-13.2 μm).

T. semipenetrans has been reported from the roots of various plants like citrus (Fars, Mazandaran, Golestan, Lorestan, Khuzestan, Kerman, Boushehr and Hormozgan provinces), olive (Kermanshah and Mazandaran provinces) and grape (Mazandaran and Markazi provinces) in Iran (Ghaderi *et al.,* 2012). Also, has been reported from the root samples of pomegranate in Kerman province (Rashidifard *et al.,* 2015). In the present study, this species was recovered from 19.9% of root samples from the rhizosphere of orange, sour orange, lemon and tangerine in the vicinity of Ahvaz, Abadan, Ramin, Shush, Dezful, Baghmalek, Andimeshk, Behbahan cities, Khuzestan province, Southwestern Iran.

Table 4. Morphometrics of *Tylenchulus semipenetrans* recovered from citrus orchards of Khuzestan province, southwestern Iran. All measurements are in *μ*m and in the form: mean ± s.d. (range).

Character	*Tylenchulus semipenetrans*		
	Female	J2	Male
n	8	8	6
L	328 ± 30.8 (288-366)	320 ± 22.1 (297-364)	309.5 ± 23 (286-334)
a	6.6 ± 1.3 (4.5-7.9)	28 ± 2.8 (24-32)	34.1 ± 1.9 (32-36.7)
b	-	3.4 ± 0.1 (3.2-3.5)	5 ± 0.6 (4-5.5)
St	10.5 ± 1.2 (9.5-12.5)	11 ± 0.8 (9.5-12.5)	8.5 ± 0.8 (8-9.5)
DGO	6 ± 1.9 (4-8)	-	-
Median bulb	60 ± 5 (52-64)	46.5 ± 2.3 (42-49)	40 ± 2.4 (36-41.5)
Median bulb length	16 ± 3 (14-19)	-	-
Median bulb width	14.5 ± 0.3 (13-15)	-	-
Pharynx length	103 ± 17.9 (84-132)	94 ± 5.3 (84-104)	58 ± 4.5 (51-63.5)
Basal bulb length	18.5 ± 0.8 (16-20.5)	-	-
Basal bulb width	13.5 ± 1.9 (11-16)	-	-
Ex. pore from anterior end	285 ± 31.9 (231-314.5)	177.5 ± 13 (156.5-192)	-
Ex. pore/L %	85 ± 2.9 (80.9-88.4)	54.9 ± 2.5 (52.5-59.4)	-
Vulva-excretory pore distance	16.5 ± 1.9 (13-18)	-	-
Post-vulva section width (PVSW)	16 ± 1.8 (14.5-18)	-	-
Post-vulva section length (PVSL)	25 ± 4.2 (19-28)	-	-
Swollen posterior body length	196 ± 32 (150-237)	-	-
Swollen posterior body as % of total body length	58.9 ± 9.2 (43-64.7)	-	-
Cuticle thickness at mid-body	5 ± 1.3 (3-6.5)	-	-
Neck length	3.5 ± 9.8 (30-52)	-	-
Vulval body width	33 ± 7.7 (24-44)	-	-
Anterior end to nerve ring	-	61.5 ± 2.4 (57-63.5)	-
Excretory pore genital Primordium distance	-	25.5 ± 3.1 (22-29.5)	-
Anterior end to genital Primordium	-	205 ± 18.2 (183.5-230)	-
Genital primordium to Posterior end	-	115 ± 12.2 (93-135)	-
Genital primordium (%)	-	64 ± 3.2 (61.2-71.1)	-
Body width at mid-body	50.5 ± 10.7 (37-64)	12.5 ± 0.6 (12-13)	9 ± 0.9 (8-10)
Anal body width	-	-	7 ± 0.7 (6-8)
Spicules	-	-	18.5 ± 1.5 (16-20)
Gubernaculum	-	-	4.5 ± 0.6 (4-5.5)
Tail	-	-	33.5 ± 3.7 (28.5-38)

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Καταγραφή φυτοπαρασιτικών νηματωδών σε εσπεριδοειδώνες της επαρχίας Khuzestan, Νοτιοδυτικό Ιράν

P. Eisvand, R. Farrokhi Nejad και S. Azimi

Περίληψη Κατά τη διάρκεια επισκόπησης για τη βιοποικιλότητα των φυτοπαρασιτικών νηματωδών στους οπωρώνες εσπεριδοειδών της επαρχίας Khuzestan (Νοτιοδυτικό Ιράν), συλλέχθηκαν 97 δείγματα ριζών και εδάφους. Από τα δείγματα απομονώθηκαν νηματώδεις, οι οποίοι ταυτοποιήθηκαν βάσει μορφολογικών και μορφομετρικών διαγνωστικών χαρακτήρων. Αναγνωρίστηκαν έξι είδη νηματωδών, συγκεκριμένα: *Helicotylenchus abunaamai, H. crenacauda, Pratylenchus allius, P. musii, Psilenchus hilarulus* και *Tylenchulus semipenetrans*. Εκτός από το είδος *T. semipenetrans*, τα υπόλοιπα πέντε είδη εντοπίστηκαν στη ριζόσφαιρα των εσπεριδοειδών, όχι στις ρίζες, και η παθογένειά τους στα φυτά δεν μελετήθηκε περαιτέρω. Αυτή είναι η πρώτη καταγραφή για τα είδη *P. allius* και *P. musii* στο Ιράν. Το είδος *H. crenacauda* αποτελεί νέα αναφορά στην επαρχία Khuzestan και αναφέρεται για πρώτη φορά σε εσπεριδοειδή στο Ιράν. Από όσο γνωρίζουμε, αυτή είναι η πρώτη αναφορά του είδους *H. abunaamai* σε εσπεριδοειδώνες παγκοσμίως.

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