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IMPROVING KEY ROOT TRAITS IN SUGAR BEET: *FUSARIUM* RESISTANCE

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GENERAL ABSTRACT

The challenge of the twenty-first century is to produce enough food to meet population demands without extending land or damaging the environment. Combining a maximum number of desirable traits such disease resistance, greater yield, and high quality is a desirable goal for plant breeders. The development of resistant crop genotypes is essential to ensure global food security, make the plant more useful and avoid crop losses. The development of molecular markers linked to the target traits is needed to predict phenotypic variation based on genotype. Marker-Assisted Selection (MAS) can reduce costs and the time required to obtain new cultivars by comparing selection only based on phenotypic evaluation. Single nucleotide polymorphisms (SNPs) are widely used as genetic marker.

Sugar beet (*Beta vulgaris* L.) is the second source of world sugar supply and is grown in all temperate zones. The crop is attacked by many pathogens and among these, the soil-borne fungus *Fusarium oxysporum* causes severe sugar beet damages. Two different *formae speciales* have been reported in sugar beet, *F. oxysporum* f. sp. *betae* that causes Fusarium yellows, and *F. oxysporum* f. sp. *radicis-betae* that causes Fusarium root rot. Disease symptoms are characterized by wilt and yellow leaves that normally die as the disease progresses. Internal symptoms consist of a brown or grey brown vascular discoloration and in the case of root rot, there is a back external rot in the primary root. Sugar beet varieties are susceptible to *F. oxysporum*, which can cause a lower root yield and reduce sugar quality. No genetic studies have been done up to now, so no genes or quantitative trait loci (QTLs) conferring resistance to *F. oxysporum* in sugar beet have been reported. The aims of this work were (i) to investigate the response of a wide collection of sugar beet lines to *F. oxysporum* f. sp. *betae*, (ii) to identify resistant lines suitable for future breeding efforts and (iii) to discover molecular markers linked to the *Fusarium* resistance that could be considered for use in marker-assisted selection (MAS) programs.

The first part of the thesis is a literature review of sugar beet breeding achievements, including the discovery of monogermity and cytoplasmic-genetic male sterility (CMS) that allowed the release of hybrid varieties. The review also focused on the breeding progresses against diseases obtained with classical and molecular methods using sources of resistance from wild beets. Next-generation sequencing (NGS) technologies with the recent release of the full sugar beet genome sequence are also

reported. Incorporation of genomics into conventional sugar beet breeding programs is essential to obtain important yield achievements in sugar beet.

The second part was aimed at screening a wide range of sugar beet lines to identify the different effect to *F. oxysporum* f. sp. *betae* inoculation and to select resistant and susceptible lines. To achieve this, 29 sugar beet lines were screened under greenhouse conditions with two highly virulent isolates belonging to different genetic sub-groups.

The third part regards an experiment conducted to evaluate the response of different sugar beet breeding germplasm to isolates of *F. oxysporum* f. sp. *betae*. In the previously tested lines, an unusual root rot was observed, normally reported in cases of infection with *F. oxysporum* f. sp. *radicis-betae.* Eight susceptible lines, from USDA-ARS (US) and UNIPD (University of Padova, Italy), were inoculated with three different isolates of *F. oxysporum* f. sp. *betae*, the causal agent of Fusarium yellows. All inoculated lines developed disease symptoms, but severe root rot was observed only in the susceptible UNIPD lines inoculated with isolates that had never caused root rot in the USDA germplasm. In this work, an unusual root rot was reported for the first time that seems to be caused not only by the isolates, but is also due to a germplasm effect.

The fourth part was aimed to identify molecular SNP markers linked to the *Fusarium* resistance in sugar beet. A candidate gene approach was used on susceptible and resistant lines to achieve this goal. Five resistant gene analogues were screened by means of a high-resolution melting (HRM) analysis and two allelic variants, within two genes, were significantly associated to *Fusarium* resistance. Sanger sequencing allowed the discovery of two SNP markers linked to the resistance. These two SNPs were significantly associated with the resistance and were mapped on the exon of *Bv7_171470_ojty* and *Bv2_043450_zhxk*, respectively.

RIASSUNTO GENERALE

Il miglioramento genetico delle piante coltivate, basato sull'esplorazione, sull'utilizzo delle risorse genetiche e sulla ricerca genomica avanzata, è prioritario per soddisfare il fabbisogno alimentare di una popolazione mondiale in costante crescita. In particolare, l'introgressione di tratti desiderabili come la resistenza alle malattie e la maggior resa produttiva è fondamentale per garantire la sicurezza alimentare a livello globale. Per accelerare il miglioramento delle piante è essenziale predire le variazioni fenotipiche sviluppando marcatori molecolari legati ai tratti in esame. La selezione assistita da marcatori molecolari può ridurre costi e tempi di ottenimento di nuove varietà rispetto alla selezione basata solo su variazioni fenotipiche. Fra i marcatori molecolari disponibili, le mutazioni di singola base (SNP) sono i più diffusi.

La barbabietola da zucchero (*Beta vulgaris* L.) è la seconda fonte di zucchero al mondo ed è coltivata in tutte le aree temperate. La coltura è colpita da numerosi patogeni e, fra questi, il fungo *Fusarium oxysporum* causa severi danni. Due differenti forme speciali di *Fusarium*, *Fusarium oxysporum* f. sp. *betae* (Fusarium yellows) e *Fusarium oxysporum* f. sp. *radicis-betae* (Fusarium root rot) sono state identificate in barbabietola. La malattia è caratterizzata da avvizzimento e clorosi fogliare con un progressivo deperimento delle foglie, spesso seguito dalla morte dell'intera pianta. I sintomi interni consistono in una discolorazione vascolare con imbrunimento dei fasci vascolari e, nel caso di marciume radicale, è presente un caratteristico annerimento all'esterno della radice principale.

Per il controllo del patogeno, l'impiego di fungicidi e le rotazioni colturali non sono efficaci. L'introgressione di geni di resistenza dal germoplasma selvatico è ritenuta la strategia principale per la difesa della coltura. Questo richiede lo sviluppo di marcatori molecolari legati ai geni di resistenza per la selezione assistita degli individui resistenti.

Gli obiettivi del lavoro di tesi sono stati i seguenti: (i) valutare la risposta a *Fusarium oxysporum* f. sp. b*etae* di un'ampia collezione di linee di barbabietola da zucchero (ii) identificare linee resistenti a *Fusarium oxysporum* da poter utilizzare in futuri programmi di miglioramento genetico e (iii) identificare marcatori molecolari SNP (polimorfismi del DNA a singolo nucleotide) legati alla resistenza a *Fusarium* da utilizzare in programmi di selezione assistita da marcatori.

Il primo contributo del lavoro di tesi descrive lo stato dell'arte dei risultati ottenuti nel miglioramento genetico della barbabietola da zucchero. Il contributo si focalizza sui

progressi ottenuti nella resistenza a malattie con metodi di miglioramento genetici classico e con l'impiego di tecniche molecolari utilizzando come fonte di resistenza germoplasma selvatico. E' stato inoltre considerato il contributo delle nuove tecnologie di sequenziamento e del recente rilascio del genoma di riferimento al miglioramento genetico della barbabietola.

Il secondo contributo riguarda la valutazione della risposta a *Fusarium oxysporum* f. sp. b*etae* di un'ampia collezione di linee di barbabietola da zucchero al fine di identificare linee resistenti e suscettibili. Per raggiungere questo scopo sono state esaminate 29 linee di barbabietola da zucchero. Le piante sono state infettate con due isolati fungini F19 e Fob220a, appartenenti a due gruppi genetici distinti, entrambi altamente patogenici. Dopo l'inoculo, per un periodo di sei settimane, è stato attribuito, per ciascuna pianta, un punteggio da 0 a 5 in base ai vari sintomi di malattia manifestati, quali: avvizzimento fogliare, clorosi e necrosi. Successivamente, le piante sono state raccolte e le radici sono state esaminate per vedere dove era presente marciume radicale, discolorazione e quali piante invece risultavano resistenti al patogeno.

Il terzo contributo descrive la risposta di due diverse collezioni di germoplasma di barbabietola da zucchero a isolati di *Fusarium oxysporum* f. sp. *betae.* Linee suscettibili, provenienti da USDA-ARS (US) e UNIPD (Università di Padova, Italia), sono state inoculate con tre distinti isolati di *Fusarium oxysporum* f. sp. *betae*, l'agente causa di Fusarium yellows. Tutte le linee inoculate hanno sviluppato i sintomi della malattia, ma un grave marciume radicale è stato osservato solo nelle linee provenienti da UNIPD inoculate con isolati che non avevano mai causato marciume radicale nel germoplasma USDA.

Il quarto contributo riguarda l'identificazione, su geni candidati, di marcatori molecolari SNPs associati alla resistenza alla malattia. In particolare, sono stati identificati 5 analoghi a geni di resistenza (RGA) dal lavoro di Dohm et al. 2014 e sono stati analizzati tramite analisi *High Resolution Melting* (HRM) su 96 campioni delle 6 linee più resistenti e più suscettibili a *Fusarium*. Due varianti, in 2 dei geni testati, sono risultate significativamente associate ($p < 0.01$) con la resistenza a *Fusarium*. Le varianti sono state validate attraverso sequenziamento Sanger. Il sequenziamento ha permesso di individuare due marcatori SNPs. L'associazione tra questi due SNPs e la resistenza a *Fusarium* è stata successivamente validata con il metodo di genotipizzazione *Comparative allele-specific PCR* (KASPar) su 96 campioni resistenti e 96 campioni suscettibili. La frequenza dell'allele A sia per lo SNP_Bv7_171470 e lo

SNP_Bv2_043450 è risultata significativamente più alta negli individui resistenti rispetto a quelli suscettibili. Questi due SNPs potranno essere utilizzati in programmi di selezione genetica al fine di migliorare la resistenza a *Fusarium* in barbabietola da zucchero.

GENERAL INTRODUCTION

Fungal diseases

Among the main causes of crop losses ever since humans began to cultivate plants are fungal diseases (Cornelissen and Melchers 1993). Phytopathogenic fungi follow three general strategies to interact with their host plants and gain access to nutrients (Horbach et al. 2011). Necrotrophic organisms obtain nutrients from dead or dying cell of living plants, they kill host cells by synthesis of toxic molecules and consume the plant tissue decomposition (Glazebrook 2005). Biotrophic organisms establish a stable relationship with living plant cells (Gan et al. 2012) because they depend on the metabolism of the infected host cells and surrounding plant tissue, to gain nutrients from living host tissue, through specialized feeding structures called haustoria (Pieterse et al. 2009). Hemibiotrophic organisms require living plants for part of their life cycle, but then kill them at later stages of the infection (Perfect and Green 2001).

Mechanisms of defense

Plants have evolved advanced defense mechanisms to oppose pathogen invasion including the activation of a wide range of responses (Zhang et al. 2013). Resistance is determined by an array of structural barriers and proteins or other organic molecules to prevent or reduce the pathogen attack (Pieterse et al. 2009). The entry of pathogens is first hindered by the waxy cuticular layers and cell wall as well as preformed antimicrobical compounds (Zhang et al. 2013). Once pathogens penetrate the cell wall, the plant actives an innate immune system. There are two forms of this type of resistance: non-specific resistance, which is efficient against several pathogenic species or several strains of a single pathogen, and specific resistance, where one plant cultivar can resist by one or a few pathogenic strains (Kiraly et al. 2007).

The innate immune system is based on the sensitive perception of pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) (Boller and He 2009), but also the recognition of endogenous signals released after attack called damage-associated molecular patterns (DAMPs) (Boller and Felix 2009). The recognition of these molecules is mediated by pattern recognition receptors (PRRs), which are cell surface-located transmembrane receptors (Zipfel 2008). PRRs consist of extracellular

leucine-rich repeats (LRR) or lysine motif (LysM) domains. This immune response is called PAMP-triggered immunity (PTI). Recognition of PAMPs produces several responses to limit the pathogens presence such as an increase of Ca^{2+} in the cytoplasm, the production of reactive oxygen species (ROS) that cause an extracellular oxidative burst, the accumulation of callose and stomatal closure. PTI also induces salicylic acid (SA) against biotrophs, and jasmonic acid (JA) and ethylene defense hormone in response to necrotrophic pathogens (Zhang et al. 2013).

A second level of active plant immunity is called effector-triggered immunity (ETI), which perceives secreted effectors produced by pathogens that have suppressed the PTI response (Jones and Dangl 2006). During evolution plants have evolved specific disease resistance (R) genes that encode R proteins able to directly or indirectly recognize the presence or the activity of single effectors of invading pathogens (Thomma et al. 2011). The recognized effector is known as an avirulance (Avr) protein. This pairwise association, known as the gene-for-gene hypothesis, was introduced by Flor (Flor 1955) and is now firmly supported by the characterization of many R-Avr gene pairs (van der Hoorn and Kamoun 2008). Functional alleles are generally inherited as dominant characters; if a functional allele is lacking, the plant becomes infected because the recognition, and so the resistance, does not happen (Keen 1990).

A hypersensitive response (HR), a form of programmed cell death localized to infection sites that increases resistance against biotrophic pathogens, is often associated with ETI (Zhang et al. 2013). The pathogens are arrested or killed in the dead plant cells because nutrients are not available. This response can increase the susceptibility to necrotrophic pathogens that can easily access nutrients from dead tissue (Poland et al. 2009). The indirect recognition of effectors is known as the guard-hypothesis. This model explains how multiple effectors could be perceived by a single R protein and how relatively few R genes can target a broad spectrum of pathogens that attack plants (van der Hoorn and Kamoun 2008).

R genes have been shown to encode two broad categories of leucine-rich-repeat (LRR) proteins that can be distinguished by protein domain structure and site of pathogen perception (Jones and Takemoto 2004). The R proteins are cytoplasmic proteins, characterized by the presence of a conserved central nucleotide binding site (NBS) and a more variable C terminal LRR domain (Collier and Moffett 2009, McHale et al. 2006). NBS-LRR proteins have been shown to function in resistance by signaling only in response to the pathogen. A second category of R proteins is inserted in the

plasma membrane and minimally consists of an extracellular LRR domain and a transmembrane (TM) domain (Jones and Takemoto 2004). Some of these transmembrane LRR proteins also have an intracellular protein kinase (PK) domain and belong to the larger class of receptor-like protein kinases (RLKs). The extracellular LRR domain of LRR-TM and LRR-TM-PK proteins is thought to function as the receptor for one or more extracellular pathogen-derived signals (Diener and Ausubel 2005).

The R genes resistance is also called qualitative resistance, whereas the other category of plant disease resistance is known as quantitative disease resistance (QDR) and is conferred by multiple genes of partial effect (Zhang et al. 2013). R genes usually confer high levels of resistance and are easy to manipulate for research and breeding improvement. However, they have some important limitations as the resistance can breakdown, caused by the strong selection and pathogen evolution. QDR is conferred by qualitative resistance loci (QRL) and is relatively broad in spectrum and robust against the pathogen evolution. QRL are involved in several biological activities in plants, such as the regulation of some morphological and developmental traits, the development of basal defense and the regulation of defense signal transduction (Poland et al. 2009).

After some local infections, plants can develop a long-lasting enhanced resistance to a broad spectrum of pathogens throughout the plant. Systemic acquired resistance (SAR) and induced systemic resistance (ISR) are two forms of systematic resistance (Boller and Felix 2009), both effective against a broad spectrum of plant pathogens (Pieterse et al. 2009). Salicylic acid has been identified as an endogenous signaling molecule that acts during SAR development and the plant protein NPR1 is necessary to transduce the SA signal for SAR (Wang et al. 2005). ISR is typically induced by soil-borne microorganisms and it is regulated by jasmonic acid- and ethylene- dependent pathways (Pieterse et al. 2009).

Fusarium oxysporum

Fusarium oxysporum Schelecht. is a ubiquitous soil-borne fungus that includes pathogenic and non-pathogenic strains (Appel and Gordon 1996). Pathogens can induce vascular wilt or root rot on a wide range of plants, causing severe crop losses (Olivain and Alabouvette 1999, Kroes et al. 1998). Pathogenic *F. oxysporum* also causes some human infections that can be superficial or limited to single organs

(Nelson et al. 1994) and disseminated infections in immunocompromised patients (Nucci and Anaissie 2007). All strains of *F. oxysporum* can be saprophytic (Leslie and Summerell 2006).

Fusarium oxysporum produces three types of asexual spores: microconidia, macroconidia and chlamydospores (Nelson et al. 1981). In *F. oxysporum* macroconidia are multicelled, thin-walled with normally three septa and a short apical cell (Leslie and Summerell 2006). Macroconidia are falcate and are often produced in a structure called sporodochium on the surface of infected plants, but also in the aerial mycelium (Leslie and Summerell 2006). Microconidia have no septa and they can have different shapes: oval, elliptical or kidney shaped and are produced in false heads (Leslie and Summerell 2006). Chlamydospores have a thick wall and are produced in the hyphae or conidia through the condensation of their contents (Nelson et al. 1994). These asexual spores have an important part in the disease cycle of *F.oxysporum*: microconidia and microconidia are involved as secondary inocula, and chlamydospores survive a long time in the soil when a suitable host is not available (Ohara and Tsuge 2004).

Fusarium oxysporum can be dispersed by many different means such as animals and wind, but also agricultural practices also play an important role in the dispersal of pathogenic strains by moving infected soil, water and by infected planting material or seed (Nelson et al. 1994). Asexual reproduction in *F. oxysporum* is done by microconidia and macroconidia while a sexual stage has never been observed (Kistler 1997).

The identification of *F. oxysporum* has commonly been based on morphological criteria such as the characteristic shape and size of microconidia and macroconidia, structure of conidiophores, formation and disposition of chlamydospores, colony color (Edel et al. 1997). On potato dextrose agar medium, strains produce mycelium that can have a highly variable color, from white to pale violet and it may be floccose, thin or abundant (Leslie and Summerell 2006).

Isolates have been divided into more than 120 different *formae speciales* based on the host or group of hosts they attack (Armstrong and Armstrong 1981). A particular *forma specialis* (f. sp.) can be subdivided into physiological races based on their virulence to a set of differential host cultivars (Correll 1991). *F. oxysporum* strains have been also grouped into vegetative compatibility groups (VCGs) based on their capacity to form heterokaryons (Puhalla 1985). This group characterization is based on the genetic of the fungus, and can be helpful because only isolates of *F. oxysporum* closely related

genetically could be in the same VCG, and since *F. oxysporum* does not have a sexual stage, strains linked by clonal descent are in the same VCG (Kistler 1997).

Many genetic markers such as isozymes (Bosland and Williams 1987), polymorphisms in mitochondrial DNA (mtDNA) (Jacobson and Gordon 1990, Kim et al. 1992), nuclear DNA polymorphisms (Assigbetse et al. 1994, Grajal-Martin et al. 1993) have been used to distinguish *formae speciales* and races of *F. oxysporum*. Phylogenetic studies suggest that some *formae speciales* in *F. oxysporum* are usually nonmonophyletic, and have originated independently during evolution (Baayen et al. 2000, O'Donnell et al. 1998). Researches have demonstrated that nonpathogenic strains of this fungus may become pathogenic by acquiring, through horizontal genetic transfer, pathogenicity chromosomes from pathogenic strains (Daboussi and Langin 1994, Jiménez-Gasco et al. 2010).

Fusarium oxysporum is considered to be a hemibiotroph (Thaler et al. 2004) because the initial stages of interaction by this pathogen are thought to be biotrophic, but later stages resemble the lifestyle of necrotrophic pathogens (Kidd et al. 2011).

The process of colonization and infection by *F. oxysporum* has been studied using light, fluorescence and electron microscopy (Di Pietro 2003, Lagopodi et al. 2002, Li et al. 2011). Dormant chlamydospores are stimulated to germinate (Nelson 1981). After germination of conidia, infection hyphae adhere to the host root surface and start to penetrate the host plant directly or through wounds (Bishop and Cooper 1983, Rodriguez-Gàlvez and Mendgen 1995), then the fungus moves to the vascular tissue developing xylem vessel elements. In infected xylem vessel elements, microconidia are produced that expand throughout the host plant (Nelson 1981). Brown discoloration is the first internal symptom that suggests *F. oxysporum* infection. The foliar symptoms in plants are vein-clearing, wilting, chlorosis and necrosis. The infected plants can become stunted and lose productivity, while in severe conditions can wilt and die (Nelson 1981).

During root penetration and colonization, *F.oxysporum* secretes many cell wall-degrading enzymes such as cellulases, xylanases, polygalacturonases, which permit the fungus access to the vascular system and then to diffuse through the xylem vessel (Roncero et al. 2003), but how these enzymes contribute to infection is not yet fully understood (Michielse and Rep 2009).

Fusarium oxysporum is exposed to different plant defense responses (Di Pietro 2003, Michielse and Rep 2009). Recognition of the pathogen induces active defense

mechanisms including physical barriers, like the plant cuticle and cell wall barriers and the production of antimicrobial compounds such as beta-1,3 glucans and phenolics (Benhamou et al. 1994), which degrade the fungal cell walls (Mauch et al. 1988). These proteins and compounds could be induced in both susceptible and resistant plants, but the resistance response is due to the difference in timing and abundance (Swarupa et al. 2014). Fungal elicitors then induce the production of lignin and phenolic compounds (Mandal and Mitra 2007), that can strengthen the cell wall (Michielse and Rep 2009).

In several crops, dominant plant resistance (R) genes have been found against host-specific pathogenic races of *F. oxysporum* (Simons et al. 1998, Oumouloud et al. 2008, Sharma and Muehlbauer 2007). In *Arabidopsis* Col-0 six dominant resistance to *Fusarium Oxysporum* (RFO) loci were observed and the locus RFO1 was identified that encoded a receptor-like kinase (Diener and Ausubel 2005). In tomato, six *Immunity* (I) loci were identified that are R-genes, which give resistance to different *F. oxysporum* f. sp. *lycopersici* races; these loci encode for proteins containing NBS-LRR domains (Sela-Buurlage et al. 2001). *Fom*-1 and *Fom*-2 are two dominant resistant genes identified in melon. The *Fom*-2 gene belongs to the NBS-LRR resistance genes (Joobeur et al. 2004). The gene-for-gene theory between *F. oxysporum* races and host cultivars is confirmed in tomato where a resistance gene *I*-3 resistance was found to *F. oxysporum* f.sp. *lycopersici* race 3, and an avirulence gene *avr*3 that breaks the resistance (Rep et al. 2005).

Molecular markers and QTLs linked to *Fusarium* resistance genes have been identified in several crops (Wang et al. 2011, Ulloa et al. 2011, Lv et al. 2013) and they can be used in plant breeding through marker-assisted selection (MAS) to improve the selection of resistance to *Fusarium*.

Fusarium yellows and Fusarium root rot in sugar beet

Fusarium oxysporum has been reported to cause two devastating diseases in sugar beet, Fusarium yellows and Fusarium root rot (Harveson and Rush 1997).

Fusarium yellows was first described by Stewart from symptomatic sugar beets in Colorado (Stewart 1931). The disease has been observed in the western United States for many years and now is becoming an increasing problem in many growing areas (Harveson and Rush 1997, Windels et al. 2005, Hanson 2006, Hanson and Jacobsen 2009).

The disease is characterized by foliar wilting and interveinal yellowing, with a vascular discoloration of the taproot (Stewart 1931). Foliar symptoms generally start in the older leaves as intervenial chlorosis (Franc et al. 2001). Often only half of the leaf will show symptoms first, followed later by the entire leaf (Franc et al. 2001). The leaves may change from yellow to brown (Hanson and Jacobsen 2009). On plants grown for seed it was reported that the seed stalk can be blighted (MacDonald and Leach 1976).

Fusarium yellows does not cause external root symptoms (Hanson and Jacobsen 2009). The internal symptoms are characterize from a brown discoloration in the vascular elements (Hanson and Jacobsen 2009) that in severe cases may spread to a rot of the vascular areas in the cortex (Hanson and Jacobsen 2009)

Fusarium root rot, which shows the same foliar symptoms, is characterized by a black rot at the distal end of taproot (Martyn et al. 1989). The disease was first reported in Texas in 1989, and was one of the major causes of sugar beet yield losses in that state (Martyn et al. 1989).

Both Fusarium yellows and Fusarium root rot can cause significant reduction in root yield, as well as reduced plant population, sucrose percentage and juice purity in affected sugar beet (Hanson and Jacobsen 2009). Disease development is favored by high temperatures (Harveson and Rush 1998) and in areas of fields that are low and compacted (Hanson et al. 2009).

The causal pathogen of Fusarium yellows in sugar beet was identified as *Fusarium oxysporum* f. sp. *betae* (*Fob*) (Snyder and Hansen 1940, Ruppel 1991), whereas another forma specialis, *F. oxysporum* f.sp. *radicis-betae* is reported to be cause root rot (Harveson and Rush, 1998).

Up to now numerous studies have been conducted to characterize the genetic diversity and evolutionary origin of *F. oxysporum* f. sp. *betae*, including vegetative compatibility grouping (VCG) (Harveson and Rush 1997), restriction fragment length polymorphism (RFLP) (Nitschke et al. 2009), random amplified polymorphic DNA markers (RAPDs) (Cramer et al. 2003), and comparisons of DNA sequences from conserved genomic regions (Hill et al. 2011). These techniques have been helpful to distinguish *F. oxysporum* f.sp. *betae* from other *Fusarium* spp., but it is still difficult to understand the pathogenic and phylogenic relationship between the *F. oxysporum* populations in sugar beet (Webb et al. 2012). Hill et al. (2011) grouped the *Fob* isolates in three clades based on the region where they were collected. The difficulty in categorizing isolates of *Fob* is also due to cross pathogenicity. In fact, *Fob* isolates can

infect other hosts, such as spinach (*Spinacia oleraceae*) (Armstrong and Armstrong 1976), various weed species (MacDonald and Leach 1976) and common bean (*Phaseolus vulgaris*). Some F. *oxysporum* isolates from spinach can even cause Fusarium yellows in sugar beets (Hill et al. 2011).

The use of resistant cultivars is an effective means to manage many diseases of sugar beet (Biancardi 2005) and genetic resistance is the primary instrument for controlling Fusarium diseases (Hanson and Jacobsen 2009).

Fusarium oxysporum resistant sugar beet genotypes are known, but the genetic systems that control *Fusarium* diseases are still unclear. Larson et al. (2007) examined the protein changes associated with a resistant and a susceptible sugar beet to *F. oxysporum,* identifying some specific resistant and susceptible specific proteins. Therefore, is essential to develop resistant cultivar and the steps to achive this goal are: to conduct genetic studies of *Fusarium* resistance, to evaluate sugar beet varieties for resistance to *Fob* using different genetic backgrounds and to develop molecular markers linked to *F. oxysporum* resistant genes in sugar beet.

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GENERAL AIMS

Aims of the thesis were:

(i) To screen a wide collection of sugar beet lines for *Fusarium* tolerance using different *Fusarium oxysporum* isolates under greenhouse conditions and identify resistant and susceptible lines.

(ii) To study the host-pathogen interaction in different sugar beet breeding germplasm with *Fusarium oxysporum.*

(iii) To identify molecular markers linked with the *Fusarium* resistance to use in marker assistant selection.

CONTRIBUTE 1

State of art of sugar beet breeding

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ABSTRACT

Sugar beet (*Beta vulgaris* L.) is an important biennial arable crop for sugar and biofuel production grown in the temperate areas. It is derived from a wild beet (*Beta maritima* L.) but is relatively a new crop and the last type to be cultivated. Sugar beet breeding began on the early nineteenth century to mainly improve root and sucrose yield. Important progresses were also obtained during last century with the discovery of monogermity, male-sterility and genetic resistances to diseases. This manuscript presents the current state of the art of sugar beet breeding with an overview on achievements obtained with classical and molecular approaches. Among them, several linkage maps have been developed to identify molecular markers associated with resistance to biotic and abiotic stresses. The use molecular markers for assisted selection led to development of genotypes with higher adaptability to adverse environmental conditions. Transgenic breeding methods for sugar beet improvement for several traits as herbicide tolerant and rhizomania are also reported. Lastly, the manuscript reports the current knowledge on available genomic resources and its application in sugar beet breeding. Incorporation of genomics into conventional sugar beet breeding programs is necessary to continue the achievement of important yield progress in sugar beet.

KEYWORDS: germplasm, wild beet, resistance traits, breeding methods, genomic resources

INTRODUCTION

Sugar beet, like chard, spinach beet, red beet and fodder beet is derived from a wild beet (*Beta maritima* L.) but is relatively a new crop and the last type to be cultivated (Coons 1936). Using mass selection, perhaps on fodder beet or natural hybrids of *Beta maritima* x fodder beets (Achard 1803, Knapp 1958), Achard obtained in 1802 the "Weisse Schlesische Rübe", the first sugar beet variety (Fischer 1989). The sugar content (S%) (sucrose percentage of root) averaged about 7%, but further selections led to a rapid improvement and in 1850 the variety "Imperial Rübe" reached values of approximately 13%. Towards 1910, cultivated varieties had sugar contents similar to

the current value of 20% (Draycott 2006) and this progress was accompanied by outstanding improvements in sugar yield.

In 1856 the first private center for beet selection was the KWS, established at Kleinwanzleben (Germany). Subsequently new private seed companies were estabilished. The list includes Van der Have (1879) in the Netherlands, Dippe (1850), Strube (1877) in Germany and Florimond Desprez (1856) in France. In the early 1900s various public research institutes started working on sugar beet selection, the first one was the Sugar Beet Experimental Station at Rovigo (Italy) founded by Munerati in 1914. After some years, the USDA Stations came into operation and were obtained important results. Crossing sugar beet with *Beta maritima,* resulted in the identification of the first resistances to cercospora, curly top and later rhizomania (Biancardi et al. 2012).

Towards the mid-1800s, the French breeder de Vilmorin achieved significant progress in breeding using progeny selection (Bosemark 2006). Instead of evaluating and selecting single plants, as is done with mass selection, single offspring from parental cross was selected and tested. Lines showing the best root yield, sugar content, polarimetric degree (purity) and other useful traits were identified and seed from these improved lines was pooled to produce a superior gene pool. This method was used for traits with low heterotic effects (Bosemark 2006).

Up to the early 1960's, the main objective of sugar beet breeding was the development of varieties with the maximum sugar yield at the lowest economic costs (Knapp 1958) through improvement of the weight and shape of the roots, sugar content, tolerance and/or resistance to diseases and bolting resistance.

Around 1970 rhizomania was becoming a significant and widespread problem affecting sugar yields. This led to increased efforts to develop resistant varieties the first of which were released some years later. At the same time increasing attention was given to the qualitative traits of the roots in order to improve the sugar extraction rates in the factory. In addition to increasing sugar content, routine analysis for sodium, potassium, alpha-amino nitrogen, the main components that reduce the processing quality of roots, was introduced in breeding stations and sugar factories (Smith et al. 1977).

Up to about 1960 the basic genetic structure of the first sugar beet varieties, apart from yield improvements, remained the same after which, thanks to the advances of breeding techniques, genetics and the cultivated varieties changed radically.

POLYPLOIDY

Sugar beet cells normally have 18 chromosomes (2n), like *Beta maritima* (Bosemark and Bormotov 1971). In 1937, Blakeslee discovered the mutagenic properties of colchicine, an alkaloid that can modify the chromosome number during the mitosis (Acquaah 2009) and in the following year, Schwanitz produced the first tetraploid (4n) sugar beet, i.e. with double set of chromosomes (Schwanitz 1938, Rasmusson and Levan 1939). 4n families, compared with 2n families, have better root shape and fewer but larger leaves with shorter and stronger petioles (Bosemark 2006). Flowers, seed clusters and pollen grains are also larger. The negative aspects are a slower seed germination and root development and breeding 4n genotypes is more difficult and time consuming than breeding diploids (Bosemark 2006).

In Europe, between 1951 and 1954, a number of polyploid multigerm varieties were produced crossing 2n and 4n lines, obtaining variable percentages of diploid, triploid and tetraploid seed. Triploid varieties, with heterosis effects, manifested intermediate morphological characteristics and were more productive than the other ploidy levels (Peto and Boyes 1940). Around the late 60's, polyploid varieties were supplanted by the better yielding hybrid varieties.

MONOGERM SEED

Originally, cultivated sugar beet varieties were multigerm (Klotz 2005). Between two and five flowers fused together to create a multigerm seedball, called glomerule. When a glomerule was planted it resulted in the emergence of multiple seedlings very close together, that immediately started to compete for light, water and nutrients. Laborious and expensive manpower was required to manually thin the seedlings in order to leave approximately 10 isolated plants per square meter (Knapp 1958). To reduce costs and work ,"technical" monogerm seed was introduced, obtained by machines able to separate the glomerules in their monogerm components. Using this method, hand thinning was reduced, but not completely eliminated, because some percentage of bi-germ seed with lower germination ability was still present (Knapp 1958).

In 1948 genetic monogermity was discovered by Savitsky, an American breeder of Russian origin. Among flowering beets of the variety Michigan 18, he found five plants

bearing single flowers, which produced seed with just one embryo (Savitsky 1950). The plant carrying monogerm flowers could be distinguished by the presence of either a lateral branch or a single flower, but never both as normally happens in a multigerm plant (Biancardi and Skaracis 2005). Selection led to the development of genetic monogerm line SLC 101, that some American and European seed companies crossed with their multigerm genotypes to obtain new monogerm lines adapted to the respective cultivation environment after 2-3 cycles of selection (McFarlane 1971). Initial problems caused by poor germination, sugar yield, and diseases resistance were reduced or overcome by means of backcrosses (Biancardi and Skaracis 2005).

Monogermity is controlled by the recessive allele *mm*, which in turn is influenced by some modifier genes that tend to slightly reduce the trait expression (Knapp, 1967). Currently, only genetic monogerm varieties are used, except in countries where the field emergence is difficult and/or manpower costs are still low, such as in some areas of Northern Africa and China.

MALE STERILITY

The release of completely hybrid varieties became possible after the discovery of cytoplasmic-genetic male sterility (CMS) (Owen 1945). Male sterile plants are characterized by flowers with white anthers that do not contain pollen. This trait derives from the interaction of two recessive genes *xx zz* with a sterile cytoplasm S. These plants are known as cytoplasmic male steriles (CMS) and to produce male sterile progeny it is necessary to cross CMS plants with a maintainer line (called an O-type) which possesses the recessive genes *xx zz*, but has a normal cytoplasm (N). In fact, if the O-type individuals are selfed, they generate 100% fertile plants, whereas when crossed with CMS plants they produce 100% sterile plants. To develop the O-types and their equivalent CMS, the O-types are selfed several times and backcrossed several times with a CMS line (Bosemark 2006).

Seed production of monogerm hybrid varieties is more complicated than for open pollinated or triploid varieties. Essentially, CMS parents (usually an F_1CMS parent) are crossed with a selected pollinator. Seedlings of both CMS and pollinators are established in a nursery in August for vernalisation through the following winter. The seedlings, known as steckles, are lifted from the nursery and transplanted in the seed production field in the following February-March, generally in a pattern of two rows of

pollinators to every six rows of CMS seed bearers. A 3:6 ratio may also be used. The transplanting costs can be eliminated sowing pollinator and CMS directly in the seed production field. After flowering, the pollinators are eliminated, the seed on the CMS plants is allowed to mature and is harvested around the end July. It is sold as a commercial variety in the following spring after processing and appropriate quality checks in seed factory. Usually the seed is pelleted to protect against pests and some fungal diseases as well as improving uniformity of sowing in the field (Winner 1993).

An alternative type of sterility is genetic nuclear male sterility (NMS), which is controlled by the expression of a single recessive gene *a* (Owen 1952). Plants with an *aa* configuration are sterile and phenotypically identical to CMS plants. NMS is used only in certain breeding procedures because it is not suited for commercial seed production (Bosemark 1971).

The release of completely hybrid varieties became possible after the discovery of CMS (Owen 1945). The first monogerm hybrid varieties used in Europe were triploids and showed a 10% improvement in yield over their contemporary diploid ones. Moreover, in the quantitative traits, they showed stronger resistance to disease (Nakamura et al. 1992) as has been verified for *Rhizoctonia* (Hecker and Ruppel 1976) and *Cercospora* (Skaracis and Smith 1987). In the last two decades triploid varieties have gradually disappeared, mainly due to the more efficient breeding procedures possible using diploid genotypes.

SELF-STERILITY AND SELF-FERTILITY

Sugar beet is an allogamous and outcrossing species due to the combined actions of protandry and a complex gametophytic self-incompatibility system (Panella and Lewellen 2007). Therefore normal families are usually self-sterile (or self-incompatible), a character that reduces or avoids inbreeding through self-fertilization (Bosemark 1993). Self-sterility is usually caused by limited growth of the pollen tubes inside the pistils preventing fertilisation of the egg (Savitsky 1950). However, other physiological mechanisms that also ensure self-sterility are the death of zygotes or the unusual growth and degeneration of embryos. Larsen (1977) established that the self-sterility in sugar beet is controlled by four gametophytic S-loci with complementary interaction, i.e. four S genes in the pollen have to match, in allelic form, genes in the pistil to prevent self-fertilization. Self-sterility was an important trait used to enhance

and maintain heterosis in multigerm varieties before the discovery of male sterility (Owen 1942).

Self-sterility is a rather unstable character, especially under particular climatic conditions or during the late flowering (Owen 1942, Bosemark 1993). Indeed, in high or low temperatures normally self-sterile plants can produce self-fertile seed. This pseudo-compatibility, due to the breakdown of the gametophytic self-incompability system is still not completely understood (De Biaggi 2005). It was noted that putting the self-sterile plants under isolation in a temperate environment as found in England, 70% of plants always produce few seeds. If the same operation is repeated in warmer environments, such as Italy, where the temperature within the isolators can reach 35-40 °C, the percentage of plants that do not produce selfed seed is significantly reduced.

Interestingly self-fertility, controlled by the dominant self-fertility gene S^F (Owen 1942), also exists in sugar beet and was demonstrated in the monogerm line SL101. Therefore plants homozygote or heterozygous for S^F do not cross with other plants (Savitsky 1950) and even under field condition self-fertile sugar beet are able to produce 90-95% of selfed seed. In breeding works self-fertility has been used with monogenic male sterility to develop recurrent populations (Bosemark 1971).

GENETIC RESOURCES

Early sugar beet breeding programs focused on developing germplasm characterized by high productivity. As production spread to different parts of the world new diseases arose, compromising production (Panella and Lewellen 2007). Few source of resistance were known for these diseases and breeders started to seek disease and insect resistance from exotic and wild beet (Panella and Lewellen 2007).

All species belonging to the section *Beta*, including *B. vulgaris* ssp. *maritima*, known also as sea beet, are cross-compatible to different degrees allowing the introduction of genetic material from one beet species to another (Bartsh 2010). Wild beet populations have a wide geographical distribution along the northern Atlantic coastline, Mediterranean basin and coasts in northern Europe (Letschert et al. 1994). At the beginning of the 20th century wild (*Beta vulgaris* ssp. *maritima*) germplasm was introduced into the sugar beet breeding pool. The first successful transfer of disease

resistance was obtained by Munerati (1913) when he back-crossed a wild beet population from Po estuary with sugar beet to introduce resistance to *Cercospora beticola*. This germplasm has been widely used in several sugar beet breeding programs to obtain Cercospora resistant varieties (Biancardi et al. 2012).

In the 1960s the innovations in sugar beet breeding, namely the introduction of a single source of CMS and limited sources for monogermity improved production but contributed to a loss of genetic variability. Crossing with *B. maritima* was a helpful tool to increase genetic diversity and reduce the potential bottleneck (Biancardi et al. 2012). Since the 1980s several *Beta* germplasm collections were made and evaluated for productivity and disease resistance to improve our understanding of the genetic resources available in the wild germplasm (Doney and Whitney 1990, Frese et al. 2001, Luterbacher et al. 2005). The first varieties to show resistance in rhizomania diseased fields comprised material of Italian origin likely to be derived from Munerati's genotypes which also contained good Cercospora Leaf Spot (CLS) resistance (Biancardi et al. 2002). When rhizomania was identified in California, several *Beta* genetic resources were screened and the second resistance gene, *Rz2*, was found in a wild beet accession WB42, collected in Denmark (Scholten et al. 1996).

Successful transfer of disease resistance from wild beet to sugar beet have been obtained for virus yellows, beet mosaic virus, beet curly top virus and powdery mildew (Biancardi et al. 2012, Luterbacher et al. 2004). *Beta* germplasm screening has been done to identify lines with genetic resistance to nematodes and resistance sources have been introgressed into sugar beet (Savitsky 1975, Panella and Lewellen 2007).

Introgressing genes from wild relatives can be difficult because of associations with adverse traits such as the annual flowering life cycle. Poor root shape that interferes with harvesting operations, high fiber contents, elongated crowns, presence of red pigment, low sugar content and low processing quality (Biancardi et al. 2012). Several backcrosses in combination with recurrent selection are needed to eliminate undesirable traits closely linked to the gene for the desired trait. It essential to identify and protect *Beta* genetic resources to conserve the genetic diversity that could be used in future sugar beet breeding programs (Frese et al 2001) as well as developing facilities for the conservation and protection of their natural ecosystems.

BOLTING RESISTANCE

Sugar beet has a biennial life cycle where the first year is characterized by a vegetative phase where the roots produced are harvested in autumn (Biancardi et al. 2005). Usually 10 to 14 weeks after sowing and in conditions of increasing day length, some beets may initiate the reproductive stage, characterized by shoot elongation (bolting) and flower development. Bolted plants reduce harvestable sugar yields especially where the percentage of bolted plants is greater than 1-2%. Bolters are also characterized by woody stems and fibrous roots that cause problems during harvesting operations (Bosemark 2006). Today these conditions are quite rare and the normal bolting percentage is less than 0.05%.

Bolted sugar beet does produce viable pollen and seeds in their first year of life (Abou-Elwafa et al. 2012). These contribute to the future weed beet population and need to be prevented and controlled.

The annual growth habit is controlled by the dominant bolting B gene as described in commercial varieties by Munerati (Munerati 1931, Owen 1954). Both environmental and genetic factors can affect expression of the gene B (Boudry et al. 1994). In fact, in sugar beet homozygous for the *B* gene, bolting is initiated under long day period, while in heterozygous plants there is a more complex response to environment, and a period of vernalisation is also necessary to induce flowering (Büttner et al. 2010). Two additional loci, namely *B2* and *B4*, were recently identified that are associated to bolting with a dominant action (Abou-Elwafa et al. 2012, Büttner et al. 2010, Hohmann et al. 2005). Gene *B2* acts epistatically to *B* and for both a photo-thermal induction is necessary to produce bolting in homozygous recessive beets.

The life cycle of wild beets depends on latitude. In a mediterranean climate they are prevalently annual and vernalization is not required for flowering. In northern areas wild beet is mainly biennial and needs vernalization for flower induction (Melzer et al. 2014). Problems with bolting can be addressed through mass selection techniques to identify individual plants resistant to bolting. These are then used in progeny tests (McFarlane 1971). Bolting sensitivity is determined by sowing seed in the field 2-3 weeks earlier than normal to stimulate vernalization. In greenhouses, this effect is obtained with adapted photo-thermal treatments (Bosemark 1993).

Under adequate selection pressure, bolting resistance in sugar beet has been considerably improved, and in regions where cold temperature are possible after

emergence, sowing time has been anticipated in order to lengthen the production cycle (Westerdijk and Tick 1991).

BIOTIC AND ABIOTIC RESISTANCES

Curly top

Curly top of sugar beet is caused by *Beet curly top virus* (BCTV), a single stranded DNA geminivirus that can cause severe and widespread losses in semi-arid areas (Strausbaugh et al. 2007). Typical symptoms are the leaf curling, discoloration, and stunting, followed by the death of the young seedling under severe infections (Strausbaugh et al. 2007). The disease is vectored by the beet leafhopper *Circulifer tenellus*. Management of the virus is difficult due to the wide host range of curly top virus and the high presence of the leafhopper in the infected environments (Wintermantel and Kaffka 2006). Effective resistance was observed following mass selection of roots in highly infested fields (Coons et al. 1931) and in 1933 the first resistant open-pollinated cultivar, designated US1, was released for commercial production (Coons 1949). Several inbreeding and progeny testing cycles were conducted to improve the varieties and to obtain BCTV resistant varieties the breeders have selfed genotypes with the S^F gene and the NMS (Owen 1952).

Abegg and Owen (1936) described a partially dominant genetic factor *C*, linked to the gene for crown color *R*. Murphy and Savitsky (1952) indicated an additive resistance in F_1 hybrids under moderate BCTV infection. In case of severe BCTV attacks on susceptible genotypes, the genetic nature of resistance appeared more complex and it was suggested that two or more genes were involved in the BCTV resistance (Savitsky and Murphy 1954).

Resistance to curly top is quantitative and combining this with resistance to other diseases is difficult (Strausbaugh et al. 2006), but in the 1990s seed companies incorporating resistance to BNYVV into commercial hybrids maintained desired levels of curly top resistance (Camp et al. 2005). The genetic control of the disease was successfully integrated with, and in some cases replaced by, insecticide treatments against the vector, but even here a combination of resistance with insecticides has only shown a reduction of disease incidence and in case of young beet even the

resistant varieties can be significantly infected and damaged (Strausbaugh et al. 2008). Further improvement of the BCTV resistance is required to improve varieties and reduce the use of chemicals.

Rhizomania

Rhizomania, the most important disease in sugar beet growing areas, is caused by *Beet necrotic yellow vein virus* (BNYVV) transmitted to sugar beet roots by the soil-borne fungus *Polymyxa betae,* that were firstly identified in Italy in 1958. Rhizomania is characterized by an extensive proliferation of the rootlets giving the root a beard-like appearance that causes a reduction of taproot development and diseased beets show necrotic rings in the root tip section. The disease causes a significant loss in sugar yield and a reduction in processing quality. The virus can cause losses of up to 80% in sugar yield (McGrann et al. 2009) and in recent decades is widespread in all main growing areas (McGrann et al. 2009). Immuno-enzymatic tests (ELISA) are used to easily quantify the BNYVV content in roots. The virus genome has been studied and molecular analysis were carried out for strain differentiation. Three distinct strain groups of BNYVV were identified (A, B and P) with geographical and pathogenic distinctions among the strains (Koenig and Lennefors, 2000, Schirmer et al. 2005). Strain groups A and B were revealed by restriction fragment length polymorphism (RFLP) (Kruse et al. 1994, Suàrez et al. 1999) and the type P was later identified by single-strand conformation polymorphism (SSCP) analysis (Koenig et al. 1995). Multiplex RT-PCR techniques have been developed for the simultaneous detection and characterization of BNYVV (Meunier et al. 2003, Ratti et al. 2005). Types A and B are distributed worldwide and contain only four RNA species and group P, has been identified in Japan, France (near Pithiviers) and UK, and contains RNA 5 (Tamada et al. 1989, Kruse et al. 1994, Koenig et al. 1995, Koenig and Lennefors 2000). Partial and complete sequencing analyses have been carried out in all the BNYVV types and variations of 3-6% between A and B types have been identified, whereas among isolates within A and B types the nucleotide sequences are highly conserved (Koenig and Lennefors 2000).

The first source of rhizomania resistance was derived from Alba P, a cercospora leaf spot (CLS) resistant germplasm, multigerm variety (Biancardi et al. 2002) which showed notably higher field performance in infected conditions even before the

discovery of the disease vectors was noted (Bongiovanni and Lanzoni 1964). The resistance was classified as quantitative (Lewellen and Biancardi 1990). In 1985, a diploid monogerm hybrid that manifested a considerable resistance, was released by SES Italy (De Biaggi 1987). Later, the Holly Sugar Company isolated the "Holly" source of resistance. The two sources of resistance were recognized as monogenic and dominant and controlled by the same gene, *Rz1* (Biancardi et al. 2002). Only a few cycles of selection were sufficient for improving this highly heritable trait (Lewellen and Biancardi 1990). The single dominant resistant gene was easily introgressed into different breeding material and several rhizomania resistant cultivars were obtained (Scholten and Lange 2000).

Resistance to rhizomania has also found in several wild accessions, as WB41 and WB42 originating from Denmark and WB258 from Italy (Panella and Lewellen 2007). The resistant gene, named *Rz2*, identified in *B. vulgaris* subsp. *maritima* WB42 population, was closely linked to the *Rz1* gene. Greenhouse tests were carried out to study the inheritance of resistance to BNYVV from the Holly source and WB42 using segregating F_2 and BC families. Resistance from Holly was simply inherited, whereas inheritance of resistance to BNYVV in WB42 was more complicated. The resistance is may be based either on one (or more) dominant major gene(s) with distorted segregation or on two complementary unlinked dominant genes, both of which required for resistance (Scholten et al. 1996). A resistance gene, *Rz3*, with incomplete penetrance, was reported linked to *Rz1* and *Rz2* (Gidner et al. 2005) on chromosome 3. In the same chromosome have been identified other two novel resistance genes, *Rz4* and *Rz5* (Grimmer et al. 2007, Grimmer et al. 2008).

BNYVV resistance-breaking strains has been first reported in the Imperial Valley of California, Usa (Liu et al. 2005), and the combination of *Rz1* + *Rz2* was showed lower virus concentration in BNYVV resistance-breaking strains infection than plants with *Rz1* or *Rz2* alone (Liu and Lewellen 2007). Overcome of *Rz1* resistance has been also found in several areas in Europe where *Rz1*- resistant sugar beet plants presented severe BNYVV symptoms in fields (Koenig et al. 2008). Greenhouse and field tests showed that the *Rz1*-resistant genotype and the susceptible genotype were both highly infected by the resistance-breaking strain, whereas the plants carrying both *Rz1* and *Rz2* was still efficient against the strain (Bornemann et al. 2014). It was observed that plants were more tolerant when *Rz1* was in combination with *Rz2* or *Rz3* then alone (Gidner et al. 2005). For this reason, it is essential to pyramid several resistance genes with different mechanisms of resistance in commercial hybrids to have higher

levels of resistance and to improve the durability of a single dominant major gene for resistance (Lewellen and Biancardi 1990).

Cercospora Leaf Spot

Cercospora leaf spot (CLS), caused by the fungus *Cercospora beticola*, is a disease that damages leaves in humid temperate cultivation areas. It is characterized by circular spots that start in the older leaves (Holtschulte 2000). The infection develops necrotic lesions that enlarge and cause a more or less rapid destruction of the leaves. The disease can lead to a reduction in root weight, and sugar content but an increase of impurities and a loss of extractable sugar (Smith and Martin 1978).

Genetic resistance to CLS has been described as polygenic and quantitative (Smith and Gaskill 1970). The discovery of CLS resistant genotypes first derived from crosses initiated around 1908 using sea beet collected along the coast of the Po River Delta (Munerati 1931). Some resistant lines were released after various backcrossing to reduce the undesirable traits of sea beet and over the years diverse commercial varieties were produced thanks to continuous selections in Italy and in the USA (Panella and Lewellen 2007).

CLS resistance is controlled by at least 4 or 5 major resistance genes with variable effects depending on the intensity of infection (Weiland et al. 2004). CLS resistance still shows a partial control of the disease, mainly due to the negative correlation existing with the yield performance (Smith and Campbell, 1996) and the difficulty to introgress this multigenic resistance into high yielding sugar beet varieties. Several fungicides proved quite effective in limiting the disease. Disease control is achieved with an integrated approach of fungicides and resistance even in severe disease conditions (Skaracis and Biancardi 2000).

Beet Cyst Nematode

Cyst nematode (*Heterodera schachtii*) is considered one of the most destructive soil-borne pests of sugar beet. Under high temperature and/or intense light conditions the

infected crop develops weakly and the leaves become wilted. Nematode can be managed using nematocides, with crop rotation and resistant varieties (Uehara et al. 2010). Control of nematodes in sugar beet is becoming increasingly difficult. In fact, intervals of at least four years between beet crops is necessary to reduce the nematode multiplication rate, but this timespan is not always enough and chemical control is severely restricted (Lilley et al. 2007). The most efficient control method is the use of resistance varieties (Uehara et al. 2010).

Nematode resistance has not been found within *B. vulgaris* but the trait was identified in the *B. procumbens* section (Biancardi et al. 2012). Interspecific hybridization with *Beta procumbens* for selecting resistance was successful (Savitsky 1960, 1975, Yu 2005). Various nematode-resistant monosomic addition lines in diploid *B. vulgaris* were identified, each carrying gene *Hs1*$^{pro-1}$ on an extra chromosome fragment coming from *B. procumbens* (Sandal et al. 1997) and homozygous resistant diploid sugar beet lines have been developed from these after recurrent selection (Heijbroek et al. 1988, Lewellen 1995). The isolation of the gene *Hs1*$^{pro-1}$ enhanced the possibility to transfer the resistance to high yielding varieties (Cai et al. 1997). However, commercial varieties containing this translocated fragment often exhibit negative phenotypic effects in the absence of severe nematode infections (Jung and Wyss 1999).

Polygenic recessive resistance to cyst nematode was found in *B. maritima* and several sea beet accessions with partial resistance were crossed with sugar beet (Panella and Lewellen 2007). The bulked F_2 were also subjected to mass selection at Salinas under rhizomania conditions to produce a broadly based sugar beet x sea beet population called R22 and only in a later stage was resistance of this line to beet cyst nematode suspected. Greenhouse tests determined the superior performance of R22, showing sugar yield was significantly inversely correlated with cyst counts (Lewellen and Pakish 2005).

Varieties carrying the resistance derived from *Beta procumbens* and *Beta maritima* have been released in USA and Europe and under field conditions, partial resistance demonstrated by a considerably reduced number of cysts has been reported (Lewellen and Pakish 2005)

Fusarium yellows and Fusarium root rot

Fusarium yellows and Fusarium root rot are caused by the ubiquitous soil-borne fungus *Fusarium oxysporum* (Harveson and Rush, 1997). The disease can cause significant reduction in root yield, sucrose percentage and juice purity in affected sugar beet (Hanson and Jacobsen 2009).

Typical symptoms are foliar wilting and interveinal yellowing, with vascular discoloration of the taproot for Fusarium yellows (Stewart 1931) and black rot at the distal end of the taproot for plants affected by Fusarium root rot (Martyn et al. 1989).

Fusarium yellows was first described in 1931 from symptomatic sugar beets in Colorado (Stewart 1931). The disease has been observed in the western United States for many years and now it is becoming an increasing problem in many growing areas (Harveson and Rush 1997, Windels et al. 2005, Hanson 2006, Hanson and Jacobsen 2009).

Sugar beet genotypes resistant to *Fusarium oxysporum* are known, but up to now there are no genetic studies of *Fusarium* resistance although Larson et al. (2007) have examined the protein changes associated with sugar beet resistant and susceptible to *Fusarium oxysporum*.

It is essential to develop molecular markers linked to *Fusarium oxysporum* resistant genes in sugar beet to conduct marker assisted selection (MAS) to overcome this increasing problem.

Abiotic stresses

The improvement of resistance and tolerance against drought, cold, heat, soil salinity etc. have been attempted by several breeders using different approaches. Considerable levels of genetic variability were observed in presence of these stresses, despite the environmental interactions (Ober and Luterbacher 2002, Stevanato 2005), and in some wild beets resistance traits were observed (Luterbacher et al. 1998).

For example, drought is a serious problem that can cause severe yield losses in semi-arid and arid region (Sadeghian et al. 2000) and between 10-30% losses in Europe

(Ober 2001, Jones et al. 2003). Sugar beet shows some resistance to dry conditions, likely because it derives from sea beet, but the potential breeding value for improving drought resistances is still unexploited due to the difficulties in transferring and introgressing this trait on high yielding germplasm. Drought tolerant varieties are important to maintain yield in different environments across years (Ober et al. 2005) and positive interaction between variety, yield and water availability has been found (Pidgeon et al. 2006). Several traits, such as leaf weight, transpiration rates and the succulence index were adopted to select tolerant genotypes (Ober et al. 2005), but they are controlled by unknown genetic factors. Similarly, for cold resistance, some degree of variance was detected in sugar beet varieties (Dix et al. 1994) and according to Wood et al. (1952), resistance to cold and to CLS appeared correlated, but until now no real improvement regarding the resistance to cold has been reported in literature. In the southern cultivation areas, temperature and light intensity are frequently excessive for sugar beet. Selection for improved tolerance of heat stress have been tried by analyzing the leaves for chlorophyll fluorescence (Clarke et al. 1993, Srivastava 1996), but no real progress seems to have been achieved. Salinity is another increasing problem in several growing area and sugar beet show useful genotypic variation for tolerance (Ober and Rajabi 2010). Breeders have still to develop effective breeding systems to contrast the increasing stresses caused by global climate changes.

Yield traits and processing quality

Sugar yield is the main trait for growers and it is defined by the root weight per hectare and the sucrose percentage present in those roots (Stich et al. 2008). The inheritance of sugar content was detailed by Savitsky (1940), who suggested two to four major genes were involved in the control of sucrose percent from crosses between divergent types such as sugar beet (15–20% sucrose), fodder and red beets (3–12% sucrose each) or chard types (12–15% sucrose). Further experiments have confirmed that sucrose percentage is a quantitatively controlled trait with high heritability, but strongly affected by the environment (Trebbi and McGrath 2003).

Sugar quality is determined by sucrose concentration and the concentration of soluble non-sucrose compounds (sodium, potassium and amino-nitrogen) that complicate the sucrose crystallization. There is a high, but negative correlation between sugar content

and root weight. Sugar content is a trait with additive variance with little expression of heterosis or dominance (Smith et al. 1973), while for root yield there is a non-additive variance (Campbell 2002). The non-sucrose compounds have a positive correlation with each other and root yield, but a negative correlation with sucrose concentration (Campbell 2002), and for that reason breeding to improve sugar quality is long and complicated process. In some cases, the non-sugar components concentration in the roots can be reduced by only a few cycles of mass selection (Powers et al. 1963, Smith et al. 1973, Smith and Martin 1989), suggesting that additive genetic variance is involved in determining the main factors of processing quality. Many of the traits that influence quality are under genetic control, but the effect of environment, cultural practices, storage conditions and so on, frequently have a greater impact (Campbell 2002).

Some anatomical and morphological traits of the roots are associated with processing quality influencing the harvest operations and the factory work (Bosemark 2006). Selection of hybrids with smooth root (with reduced or without the two vertical grooves and with smooth skin) lowers the amount of soil carried to the factory, that can cause damage during the slicing and diffusion phases (Tsialtas and Maslaris 2010). Smooth root varieties, with improved root shape and reduced crown dimension, derived from hybrids between sugar beet and garden beet (Panella and Lewellen 2007), were developed through repeated cycles of mass selection (Mesken and Dieleman 1988, Saunders et al. 1999).

Sugar beet breeders are also interested in traits related to seed multiplication of commercial varieties. Production of high quality seed is essential since seed with high germination significantly influences the field emergence and uniformity. This trait in combination with vigour of seed and seedling has significant effects on sugar yield (Biancardi et al.2010). The seedlings need to develop quickly and the leaves have to filled in the space between rows as soon as possible with a uniform plant density to increase the amount of light intercepted (Biancardi et al.2010) and reduce development and competition of weeds (Paolini et al. 1999).

MOLECULAR BREEDING ACHIEVEMENTS AND FUTURE PERSPECTIVES

Around 1990, sugar beet breeding started to be assisted by the molecular biology techniques, which made selection easier and more certain (Skaracis 2005). Molecular

markers (polymorphism at the molecular level) are used to create genetic linkage maps, genotyping and marker-assisted selection (MAS) (Biancardi et al.2010).

Genetic linkage maps

Genetic linkage maps are a powerful tool in plant breeding. Linkage maps represent the position and the relative genetic distance between polymorphic traits along chromosomes (Collard et al. 2005), Molecular markers are developed to detect polymorphisms, define the genetic distance between the traits and identify chromosomal locations of genes and quantitative trait loci (QTLs) associated with the traits of interest (Mohan al. 1997). Genetic linkage maps are based on the principle that genes and markers can segregate during meiosis and if they are close together, they can be transmitted together to the progeny more frequently than genes or markers that are more distance (Collard et al. 2005). A segregating population from parents that differ for the traits of interest are needed for the construction of linkage maps.

One of the first linkage maps in sugar beet was based on RFLP (Restriction Fragment Length Polymorphism) markers as reported by Pillen et al. (1992). The map included 115 markers (108 RFLP markers, 6 isozyme and 1 morphological trait) that covered 789 cM. Later it was extended using 117 markers (168 RFLP markers, 7 isozymes and 2 morphological traits) distributed over 1057.3 cM (Pillen et al. 1993). In both maps, markers was distributed over 9 linkage groups that correspond to the haploid chromosome number of sugar beet.

RAPD (Random Amplified Polymorphic DNA) markers were used for the construction of a genetic map in sugar beet by Uphoff and Wricke in 1995. The map included 85 RAPD markers, 5 isozymes and genes for nematode resistance, annual flowering and hypocotyl colour and covered 738 cM.

Based on the linkage map of Pillen et al. (1993), an integrated map was built using 120 AFLP (Amplified Fragment Length Polymorphism) and 207 RFLP markers that allowed the development of a high-density map (Schondelmaier et al. 1996). The markers were distributed over 9 linkage groups, covering 621 cM. A SSR (Simple Sequence Repeats) based linkage map has been constructed where 23 markers have been assigned to each of the 9 linkage groups (Rae et al. 2000). ESTs (Expressed

sequence tags) have been used to develop a functional genetic map of sugar beet (Schneider et al. 2002), since using EST collections and QTL mapping is possible to identify genes. A linkage map from a sugar beet x table beet population has been constructed using 331 markers (3 morphological, 25 RFLP, 242 AFLP 46 SSR, 14 EST and 1 STS markers) that covered 526.3 cM among the nine linkage groups (McGrath et al. 2007). Recently, SNPs (Single Nucleotide Polymorphism), that are co-dominant markers and highly abundant in the sugar beet genome, have been used to develop high density marker maps (Schneider et al. 2001, Mohring et al. 2004, Schneider et al. 2007). Schneider et al. (2001) sequenced 37 gene fragments developed from ESTs in two inbred sugar beet lines, detecting a frequency of one SNP every130 nucleotides. One SNP every 72 bp in 315 EST-derived loci was found in a panel of 13 lines of sugar beet (Schneider et al. 2007).

Identification of molecular markers

Molecular markers can be used to identify desirable traits at a very early developmental stage, saving considerable time and accelerating the breeding work (Mohan et al. 1997).

Identification of a DNA marker linked to the *Rz1* gene resistant to rhizomania was first reported by Barzen et al. (1992). The gene was mapped on linkage group 4 associated to a RFLP marker located at 6.7 cM from the *Rz1* locus. Later, 12 RAPD markers linked to the gene were selected by bulk segregant analysis (BSA) and mapped on the same linkage group (Barzen et al. 1997).

Several studies have been conducted to identify molecular markers linked to the *Rz1* gene for resistance to rhizomania (Pelsy and Merdinoglu 1996, Scholten et al. 1996, 1997). Resistant and susceptible plants were crossed to obtain segregating families and the plants were inoculated in greenhouse with infected soil (Scholten and Lange 2000). The second dominant resistant gene, *Rz2*, was mapped with RAPD and STS (Sequence Tagged Sites) markers, and it is located about 20 cM from *Rz1* (Scholten et al. 1997, 1999). Other major resistant genes have been found linked to BNYVV resistance. The resistance in sea beet accession WB41 has been studied using AFLP markers and was identified a major rhizomania resistance gene on chromosome 3, which was called *Rz3*, distant 5 cM from *Rz1* (Gidner et al. 2005). *Rz1*, *Rz2* and *Rz3* are all located on chromosome 3 and because of *Rz2* and *Rz3* derived from two sea

beets both collected in Denmark at almost the same period it is not clear if they are the same gene or not. Studying a segregation population from composite cross of *B. maritima* accessions with sugar beet with BSA using AFLP, SNP and RAPD markers allowed to identify a major QTL for BNYVV resistance, named *Rz4*, that explained 78% of the phenotypic variation and was located on chromosome 3 (Grimmer et al. 2007). The *Rz5* was identify in a mapping population using SNP and AFLP markers from the Italian WB258 accession and mapped *Rz4* and *Rz5* were located to 6.9 cM and 6.0 cM respectively on chromosome 3 both explaining more than 80% of the phenotypic variance. It was suggested that *Rz1*, *Rz4* and *Rz5* represent alleles of the same gene (Grimmer et al. 2008). Several bacterial artificial chromosome (BAC) linked to resistance gene analogues (RGAs) have been mapped on chromosome 3 using a segregating population for rhizomania resistance (Lein et al. 2006) and has been observed that 5 major gene clustering in two groups. The first locus might coincide with *Rz1*, *Rz4* and *Rz5*, while the second locus represented the *Rz2* and *Rz3*.

Fertility restorer loci *X* and *Y* were located in chromosomes 3 and 4 respectively (Schondelmaier and Jung 1997). Gene *X* was mapped with an isozyme and RFLP markers terminally on chromosome 3 at a distance of 9.6 cM (Pillen et al. 1993, Uphhoff and Wricke 1995). Two QTL regions were identified at a distance of 15 cM on chromosome 4, explaining 79% of the variability, and one QTL, mapped on chromosome 3, explained 72% of the observed variation (Hjerdin-Panagopoulos et al. 2002).

A new source of CMS (H-CMS) from wild beets has been described by Boudry et al. (1993) and the gene has been mapped to chromosome 4, applying BSA analysis and 9 RAPD markers linked to the H-CMS locus (Laporte et al 1998). The gene is also linked to the gene for the monogerm seed trait (Laporte et al 1998). Using a restorer line, NK-198, it was observed that pollen fertility segregated as a single dominant gene named *Rf1*. Two RAPD and 8 AFLP tightly linked to the gene were found that mapped to a terminal region of chromosome 3, suggesting that *Rf1* may be an allele of the locus X (Hagihara et al. 2005).

Resistance genes for beet cyst nematode (*Heterodera schachtii*) were found among wild species of section *Patellares* and 3 different resistance genes have been identified, *Hs1* on the homoeologous chromosomes 1 of *B*. *procumbens*, *B*. *webbiana* and *B*. *patellaris, Hs2* in the homoeologous groups to chromosome 7 of *B. procumbens* and *B. webbiana,* and *Hs3* in chromosome 8 of *B. webbiana* (Kleine et al. 1998). Resistance genes have been introgressed into sugar beet through

chromosome translocation (Jung and Wricke, 1987, Heijbroek et al. 1988). To identify chromosome segments with *Hs1* from wild types in segregating offspring of monosomic additional lines (2n = 19) genome specific DNA probes were used (Schmidt et al. 1990, Jung and Hermann 1991, Jung et al. 1992). Heller et al. (1996) mapped 4 wild beet translocations from independent translocation events with RFLP markers. Marker loci were used to show close linkage (0-4.6 cM) with the end of chromosome 9, suggesting a recombination hot spot in this region. *Hs1*$^{pro-1}$ gene was mapped at 3 cM to a RAPD marker at the end of chromosome 9 (Hallden et al. 1997). Two sugar beet lines carrying homologous translocations from wild beet (*Beta procumbens*) have been studied and a complete physical map of a *Beta procumbens* was done (Schulte et al. 2006). Moreover, a second nematode resistance gene *Hs1-1* $^{pro-1}$ was suggested. A nematode tolerance gene, termed *HsBvm-1* from *Beta vulgaris* ssp. *maritima* was mapped on chromosome 5 and using a BSA approach a SNP marker was found to be completely associated with the gene (Stevanato et al. 2015).

Breeding for *Cercospora* resistance is difficult because it is a quantitative trait. Studies with Quantitative trait loci (QTLs) try to identify the location of those regions of the genome where the genes are involved in the specific trait. The QTL then needs to be validated, especially when the traits have a high interaction with the environment. QTLs studies have been used to identify chromosome regions associated with *Cercospora* resistance. Five QTLs have been mapped using 221 AFLP and 46 RFLP markers with a phenotypic variation from 7 to 18%. These QTLs were located on linkage groups 1, 2, 3 and 9 (Nilsson et al. 1999). Schafer-Pregl et al. (1999) identified QTLs in F_3 families and F_2 backcrosses under natural and artificial inoculation using 224 markers (RFLP, AFLP,SCAR and microsatellites): three major QTLs were located on chromosomes 2, 6 and 9 in all conditions, three more QTLs were detected on chromosomes 4 and 5 in an F_2 population only. Using a linkage map based on AFLP and RFLP markers, a QTL analysis was done on F_2 population and F_3 half-sib families. 4 QTLs on chromosomes 3, 4, 7 and 9 have been found based on data from a leaf-disc test and 5 QTLs associated with chromosomes 4, 7, 8 and 9 based on field data (Setiawan et al. 2000).

Lines resistant and susceptible to CLS were used to generate recombinant inbred lines to identify QTLs. 4 QTL loci involved in CLS resistance were detected: 2 QTLs, qcr1 on chromosome 3 and qcr4 on chromosome 9, were found in the resistant line and explained approximately 10% and over 20% respectively, of the variance in the resistance index, while qcr2 on chromosome 4, qcr3 on chromosome 6 were found in the susceptible line, each explained about 10% of the variance. Both qcr1and qcr4

were mapped precisely as single QTLs, using progenies BC_5F_1 and BC_2F_1 obtained by recurrent backcrosses (Taguchi et al. 2011).

Root and sugar yield as well as physiological traits are inherited quantitatively. Different molecular markers were used to find QTLs for the sugar yield related traits (Weber et al. 2000, Schneider et al. 2002, Stich et al. 2008, Reif et al. 2010, Wurschum et al. 2011). Weber et al. (2000) tested two segregating populations in several environments to identify QLTs linked to these traits. A number of QTLs were detected, but they mapped on different chromosomes in the two populations and just a few QTLs were correlated in different locations. QTLs for 7 traits related to sugar quality were mapped using 75 Expressed sequence tags (ESTs) associated with functions in carbohydrate and nitrogen metabolism. The experiment was done in 6 different environments to evaluate the test-cross progeny, and the genes were mainly mapped as SNPs. 21 significant QTLs were found, all linked to ESTs (Schneider et al. 2002).

Molecular markers linked to the bolting gene are important to allow early identification of bolting plants. The first linkage maker was an isozyme marker (Abe et al. 1992) and later RFLP markers flanking the B locus were identified, with respective distances of 3.8 and 5.2 recombination units located to the center of chromosome 2 (Boudry et al. 1994). El-Mezawy et al. (2002) identified 2 AFLP markers that mapped close to the bolting gene, at a distance of 0.14 and 0.23 cM. These markers have been used for map-based cloning of the *B* gene (Hohmann et al. 2003). A second bolting locus, termed *B2*, was mapped to chromosome 9 (Büttner et al. 2010). A QTL analysis reported another new locus, *B4*, genetically linked to the *B* locus and mapped to chromosome 2 at a distance of 11 cM from *B* (Abou-Elwafa et al. 2012).

In many species, flowering is regulated by complex genetic networks. In *Arabidopsis thaliana*, flowering is regulated by FLOWERING LOCUS C (FLC), CONSTANS (CO), and FLOWERING LOCUS T (FT) and homologs of these genes have been identified in sugar beet. Reeves et al. (2007) found *BvFL1* on chromosome 6 and *BvCOL1* has mapped on chromosome 2 at distance of 22-24 cM from *B* and 35-38 cM from *B4* (Chia et al. 2008, Abou-Elwafa et al. 2012). Two paralog *FTs* have been identified, *BvFT1* on chromosomes 9 and *BvFT2* on chromosome 4, which act antagonistically (Pin et al. 2010). In fact, *BvFT1* repress flowering, while *BvFT2* promotes it (Pin et al. 2010).

Approaches using association mapping have been adopted to identify the most important QTLs in multi-trait expression (Stich et al. 2008, Wurschum et al. 2011).

Stich et al. (2008) analyzed 111 diploid sugar beet lines in 6 locations and each line was genotyped with 26 SSR markers. 4 markers associated with sugar content and beet yield were found. In another study, 924 elite sugar beet inbred lines were tested in 7 locations for sugar and root yield and non-sugar compounds (Wurschum et al. 2011). A linkage map was built using 677 SNP markers that cover the entire genome. Main effect QTLs were detected for all the traits studied, some of which were major QTLs which accounted for genotype variances of more than 5%.

High density genetic maps are helpful tools to improve sugar beet and the integration with physical maps allows QTL genes to be cloned. Many disease resistance genes have been cloned. These genes have highly conserved sequences with common functional domains, nucleotide binding site (NBS) domains and leucine rich repeat (LRR) domains, able to recognize the pathogen. Many of the genes over expressed during an infection encode for pathogenesis related (PR) proteins. Using the degenerate primer approach, resistance gene analogs (RGAs) were cloned and mapped in different genomes (Rossi et al. 2003, Collins et al 1998, Shen et al. 1998). In sugar beet 33 RGAs have been mapped (Hunger et al. 2003) and 3 RGAs were linked with *Cercospora* resistance QTLs on chromosomes 5, 7, and 9. Additionally 2 RGAs were linked with rhizomania resistance gene *Rz1,* localized on chromosome 3.

A useful tool in disease resistance breeding can be the use of molecular markers with pyramiding. The combination of several desirable genes and QTLs from different genetic backgrounds incorporated into a single genotype can be useful to develop stable disease resistance (Richardson 2010).

The evaluation of genetic diversity and population structure is essential in any breeding program and in sugar beet several studies to address this have been conducted (Kraft et al. 2000, Li et al. 2010, Li et al. 2011). Kraft et al. (2000) examined the occurrence of Linkage Disequilibrium (LD) in nine sugar beet breeding lines based on 451 mapped AFLP markers and found that pairs of markers within 3 cM distance showed high LD. Moreover, 23 SSR markers were used to examine the population structure of an elite sugar beet germplasm and to investigate genetic diversity within and among subgroups of this germplasm. Two distinct subgroups were found within the entire germplasm set by STRUCTURE and principal coordinate analysis (PCoA) and the predicted value of r^2 declined to 0.1 or less within about 10 cM (Li et al 2010). A genome-wide distribution map of genetic diversity and LD in an elite sugar beet germplasm, with 246 yield type and 238 sugar type pollen parent inbred lines, was obtained using 328 SNP markers (Li et al. 2011). Two distinct subgroups in the elite

sugar beet germplasm were identified based on different statistical methods as MCLUST, STUCTURE and PCoA. Significant LD between loci pairs was found at distances of 7 cM, 45cM, and 21 cM for the entire germplasm set and lines inbred for yield or sugar content respectively (Li et al. 2011). In a recent work, genetic diversity and LD were investigated in 233 elite sugar beet breeding line using 454 SNP markers and 91 wild beet accessions with 418 SNPs (Adetunji et al. 2014). PCoA showed three distinct groups: the wild beet, the seed parent and the pollen parent breeding lines. LD persisted beyond 50 cM on four chromosomes in the pollen parent pool, and three chromosomes in the seed parent pool. LD decreased within a distance of <6 cM on all chromosomes in both pools when genetic relatedness was corrected. Regions on chromosomes 3 and 4 where the disease resistance and monogermity loci are located showed strong genetic differentiation between the pollen and seed parent pools (Adetunji et al. 2014). These studies of LD decay can be used to design future Genome-wide association studies (GWAS).

Molecular markers can allow to analyze phylogenetic relationships, to identify parental backgrounds in hybrids, to monitor gene flow and genetic variation in populations (Skaracis 2005). Oligonucleotide DNA probe hybridization has been used to fingerprint sugar beet and identify double-haploid breeding lines (Schmidt et al. 1993). Sugar beet lines have been fingerprinted using AFLP markers (Hansen et al. 1999, Kraft et al. 2000). The development of high throughput marker systems allow a rapid genotyping. Möhring et al. (2004) fingerprinted several sugar beet breeding lines with linkage group-specific SNP marker sets. In another study, a set of 54 varieties from five seed companies was genotyped using 702 Diversity Array-Technology (DArT), 34 SNP and 30 SSR markers and analyzed for population structure. Three well-defined populations was observed and the clustering of varieties was well correlated with their seed company origin although some of the hybrid varieties likely have a parent closely related. Moreover, just 17 SSR markers were needes to identify the 54 hybrid varieties (Simko et al. 2012). When fingerprint analysis of 15 sugar beet genotypes (from CMSs, pollinators, and commercial varieties) was conducted using 192 SNP markers, PCoA and STRUCTURE analysis showed that CMSs, pollinators and varieties clustered into three distinct subpopulations (Stevanato et al. 2014).

TRANSGENIC TRAITS

Plant genetic engineering started in the 1980's with the insertion of helpful genes into plant genomes (Barton et al. 1983, Horsch et al. 1985) and researchers developed new traits to improve the crop production (Prado et al. 2014).

Sugar beet is very sensitive to weed competition at earlier stages of growth, so weed control is particular importance especially during the first three months of vegetative growth to prevent competition for light and water, to avoid significant losses (May et al. 2005). Weed managements strategies are labour-intensive and expensive because combination of multiple herbicide active ingredients are required with multiple applications (Skaracis 2005). Genetically modified sugar beets have been developed for herbicide tolerance, to allow the use of a cheap, non-selective single herbicide that can be efficiently used against a wide range of weeds. For example, a gene conferring tolerance to glyphosate, a commonly used broad spectrum herbicide, has been integrated into the sugar beet genome (Duke 2008). Sugar beet lines have also been transformed with the 5- enolpyruvylshikimate-3-phosphate synthase gene (*CP4 EPSPS*) from *Agrobacterium* sp. *CP4* and a glyphosate oxidase reductase gene (*GOX*) from bacteria, both able to confer high tolerance to glyphosate (Mannerlöf et al. 1997). In 2007, a genetically modified herbicide tolerant sugar beet, termed *H7-1*, developed by Monsanto in cooperation with KWS Saat, was introduced in the USA and Canada and since 2009, 95% of US sugar beets were glyphosate-resistant (James 2010). Weed management has been simplified with herbicide-resistant sugar beet reducing time and costs for treatments (Khan 2010).

Transgenic studies in rhizomania resistance were conducted introducing a viral coat protein (*CP*) gene from the BNYVV. First transformed sugar beet suspension cultures were obtained after cultivation of sugar beet cells with *Agrobacterium tumefaciens* carrying the *CP* gene of the BNYVV, and using protoplasts from these cells showed resistance to virus infection (Kallerhoff et al. 1990). The *CP* gene was also stable transferred and expressed in sugar beet hair root cultures using *Agrobaecterium rhizogenes*-mediated transformation (Ehlers et al. 1991). Tests of a *CP*-mediated rhizomania resistance have shown a reduced virus content in the greenhouse as well as in field trials (Mannerlöf et al. 1996). Another approach to develop resistance to viral diseases is RNA silencing mechanism, an active defense system against viral invaders in plants (Ding and Voinnet 2007) and in sugar beet this technology has been used against BNYVV (Lennefors et al. 2006, Pavli et al. 2010, Zare et al. 2015). Sugar

beets have been transformed with a RNA silencing mechanism by the expression of a 0.4 kb inverted cDNA, derived from the BNYVV replicase gene (Lennefors et al. 2006). The plants showed high levels of resistance against different genetic strains of BNYVV under high infection levels in the greenhouse and in the field. Pavli et al. (2010) used a dsRNA-mediated silencing against the BNYVV replicase gene in sugar beet with transgenic hairy roots reporting a good level of BNYVV resistance. The stabile inheritance of the transgenes and virus resistance over years has been demonstrated using both transient and stable transformation methods and resistance induced by intron-hairpain RNA constructs with small intronic loops showed highest degrees of resistant events (Zare et al. 2015).

Genetic transformation in beets has been used also to study the salt-tolerance. In *Arabidopsis thaliana*, six vacuolar Na+/H+ antiporters (*AtNHX1-6*) were identified (Aharon et al. 2003, Pardo et al. 2006), and overexpression of *NHX* genes in transgenic plants showed salt- tolerant in several species (Zhang and Blumwald 2001, Zhang et al. 2001, Xue et al. 2004). In sugar beet two genes, *AtNHX1* and *AtNHX3*, have been integrated into the genome (Yang et al. 2005, Liu et al. 2008) and in both the experiments transgenic plants showed highly improved salt tolerance. Under high salt conditions, the soluble salts were accumulated in the leaves and not in the roots, increasing root yield (Liu et al. 2008).

Sugar beet production is limited by drought stress and to improve resistance, sugar beet plants have been modified to produce fructan (Pilon-Smits et al. 1999). Fructans are water-soluble polymers of fructose able to store carbohydrate and defend plants against drought stress (Pilon-Smits 1995). Sugar beets were transformed with the *SacB* gene from *Bacillus subtilis*. The transgenic plants accumulated fructans in storage roots as well as in older leaves, showing better growth under drought conditions compared to nontransformed sugar beets (Pilon-Smits et al. 1999). Sugar beet has been transformed with 1-sucrose:sucrose fructosyltransferase (*1-SST*) gene isolated from Jerusalem artichocke (*Helianthus tuberosus*) (Sévenier et al. 1998) and from onion (Weyens et al. 2004). A higher conversion of stored sucrose into fructans was reported in both studies. Putative drought-tolerant sugar beet plants were obtained in *in vitro* tissue cultures using gamma irradiation-inducing mutation (Sen and Alikamanoglu 2012). In this work, inter-simple sequence repeats (ISSR) markers and SDS-PAGE analysis showed genetic distance between mutant and control plants.

Artificial mutant collections often show new variability not present in natural plants, and the combination of mutagenesis with targeting induced local lesions in genomes

(TILLING) is a helpful approach to identify the allelic variants present in the DNA regions of the artificial mutants (Pérez-de-Castro et al. 2012). In sugar beet the bolting gene *B* was mutated using ethylmethanesulfonate (EMS) and further characterised by TILLING several mutagenized plants showed a biennial behavior (Hohmann et al. 2005). Subsequently, F_2 populations derived from annual beets and biennial mutants have been studied showing that an EMS-induced mutation of *B* gene can be sufficient to remove the annual behavior, and a new bolting locus unlinked to the bolting gene has been identified (Büttner et al. 2010).

NEW PERSPECTIVES IN SUGAR BEET BREEDING

Huge amounts of genomic data are now available and can be used for genomic selection and accurate prediction of genomic breeding values for genotyped individuals within a breeding population (Hayes and Goddard, 2001). This type of genomic-assisted breeding has been studied in sugar beet. Würschum et al. (2013) evaluated the potential of genomic selection using a large sugar beet population of 924 lines from different germplasm sources. The lines have been phenotyped in several trials for 6 agronomical traits and genotyped with 677 SNP markers. Genomic prediction has been investigated genotyping, with 192 SNPs for root vigor, 124 plants from 18 lines (Biscarini et al. 2014). The accuracy of estimated prediction was quite high, with an estimated cross-validation error rate close to zero. Such accurate predictions may be related to a high heritability for the root vigor trait (0.783).

The full sugar beet genome sequence has recently become available (Dohm et al. 2014) and should be useful to identify genetic basis of important traits. It represents 85% of its 576-Mbp genome size and 27,421 protein-coding genes were identified supported by transcript data and based on sequence homology. The release of the sugar beet genome with supplementary data of predicted genes, their functional annotation and a list of 715 putative RGAs should be very useful tools for discovering important traits, determining the position and function of genes and developing highly accurate maps.

The emergence of next-generation sequencing (NGS) technologies allows a fast and cheap sequence of millions bases that can be used to develop new molecular markers (Egan et al. 2012). The markers from NGS can also help to accurately determine the

position within the physical maps and to create high-resolution maps (Pérez-de-Castro et al. 2012).

Dense genome coverage will allow the identification of molecular markers closely linked to specific genes and important QTLs. The large and increasingly accurate data derived from NGS and the more automated phenotyping systems need to be combined with new bioinformatics tools available to analyze the data efficiently (Varshney et al. 2014). Incorporation of genomics into a conventional breeding and integration across different scientific fields is also necessary to achieve important progress in sugar beet breeding.

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CONTRIBUTE 2

Evaluation of F*usarium oxysporum* f. sp. *betae* tolerance in accessions of sugar beet

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ABSTRACT

Fusarium oxysporum f. sp. *betae* (FOB) is an important pathogen of sugar beet worldwide causing leaf yellowing and vascular discoloration. The use of tolerant varieties is one of the most effective methods for managing this disease. In this study, a large germplasm collection, comprised of 29 sugar beet parental lines, was tested for FOB tolerance under greenhouse conditions. Five-week-old sugar beet plants were inoculated using a root dip method with two highly virulent isolates of FOB, F19 and Fob220a. Symptom severity was assessed weekly for six weeks, and the area under disease progress curve (AUDPC) was used to estimate disease development. Both isolates were virulent. Plants inoculated with F19 showed a significantly higher AUDPC ($p<0.05$) as compared to plants inoculated with Fob220a. The evaluated lines showed a varying tolerance response, from extremely susceptible to highly tolerant. Several roots from susceptible lines showed internal discoloration as well as root rot. This first screening for *Fusarium oxysporum* resistance on an Italian sugar beet germplasm led to identification of highly tolerant genotypes. Incorporation of these sources of tolerance to a breeding program will contribute to improve Fusarium tolerance in sugar beet.

KEYWORDS: Biotic stress, genetic resistance, *Fusarium oxysporum*, sugar beet

INTRODUCTION

Sugar beet (*Beta vulgaris* L.) produces 25% of the global annual sugar supply and the crop is widely distributed throughout the temperate regions of the world (Draycott 2006). Remarkable progress has been made on sugar beet breeding for agronomic traits such as root yield, sugar content and disease tolerance over the last 60 years (Biancardi et al. 2010). Many pathogens including fungi, viruses and nematodes attack sugar beet and diseases such rhizomania, Cercospora leaf spot (CLS), beet cyst nematode, Fusarium yellows and Fusarium root rot are increasing their impact on sugar beet production in many growing regions (Biancardi et al. 2010).

Fusarium oxysporum is a soil-borne fungus that induces vascular wilt or root rot on a wide range of crops causing severe losses (Olivain and Alabouvette 1999, Kroes et al. 1998, O'Donnell et al. 1998), including (i) Fusarium yellows and (ii) Fusarium root rot in sugar beet (Harveson 2009). Fusarium yellows, caused by *Fusarium oxysporum* f. sp. *betae,* was first described in 1931 from symptomatic sugar beets in Colorado (Stewart 1931), then reported in several sugar beet growing areas (Harveson and Rush 1997, Windels et al. 2005, Hanson 2006, Hanson and Jacobsen 2009). Fusarium root rot caused by *Fusarium oxysporum* f. sp. *radicis-betae* was first reported in 1989 in Texas (Martyn et al. 1989). In both cases, the fungus penetrates through the roots and grows in the vascular tissue, causing wilting in affected plants and interveinal yellowing which usually develops first on the older leaves followed by the younger leaves, in addition to vascular discoloration of the taproot (Hanson and Jacobsen 2009). The root rot has the same foliar symptoms as Fusarium yellows but the root is characterized by a black external rot on the tip of the tap-root (Harveson 2009). Fusarium diseases cause significant reductions in root yield, sugar content and purity (Hanson and Jacobsen 2009).

Chlamydospores of *Fusarium oxysporum* remain in the soil for years and thus traditional control methods are not always efficient. Chemical control is difficult to apply with a soil-borne pathogen and has a high environmental impact, and crop rotation needs a long rotation that is economical not viable (Harveson 2009). Introgression of disease resistance into sugar beet varieties is the best way to overcome this problem (Biancardi et al. 2010). To achieve this goal, it is essential to have resistant donor parents to breed resistant lines.

Host-pathogen interactions have been studied in several crops and dominant plant resistance (R) genes against host-specific *F. oxysporum* races have been identified (Simons et al. 1998, Oumouloud et al. 2008, Sharma and Muehlbauer 2007). In *Arabidopsis thaliana* ecotype Col-0, six dominant resistance loci to *F. oxysporum* (RFO) were observed, and the locus *Rfo1*, encoding a receptor-like kinase, was identified (Diener and Ausubel, 2005). In tomato, six *Immunity* (I) loci were found which are R-genes that confer resistance to different *F. oxysporum* f. sp. *lycopersici* races. These loci encode for proteins containing NBS-LRR domains (Sela-Buurlage at al. 2001). In melon, the two dominant R genes are named *Fom*-1 and *Fom*-2 and the *Fom*-2 gene belongs to the NBS-LRR resistance genes (Joobeur at al. 2004). The gene-for-gene theory between *F. oxysporum* races and host cultivars was confirmed in tomato where a resistance gene *I*-3 was found to be effective against *F. oxysporum* f.sp. *lycopersici* race 3, and also a pathogen avirulence gene, *avr*3, that breaks the

resistance was identified (Rep et al. 2005). Several molecular markers and QTLs linked to *Fusarium* resistance genes were identified in different crops (Wang et al. 2011, Ulloa et al. 2011, Lv et al. 2013) and have potential for use in plant breeding to improve the selection of resistance to *Fusarium* through marker-assisted selection (MAS).

In sugar beet, *Fusarium oxysporum*-resistant lines are known, but the genetic system that controls *Fusarium* diseases is still unclear. Developing new resistant sugar beet cultivars with resistance genes depends on an effective screening technique. The evaluation of tolerant sources under controlled conditions allows for reduced environmental effects, enhancing the genetic control of the disease symptoms shown by each line, thus allowing an effective screening. The objective of this work was to evaluate the susceptibility of a wide set of sugar beet lines to *Fusarium oxysporum* isolates under greenhouse conditions to identify potential resistant genotypes that may be used as donors in a breeding program.

MATERIALS AND METHODS

Plant materials and growing conditions

The study was performed on 29 sugar beet genotypes with 27 sugar beet pollinators from DAFNAE-Department of Agronomy, Food, Natural resources, Animals and Environment (University of Padova, Italy). A sugar beet variety, 20, provided by Lion Seeds Ltd (UK) and a resistant pollinator inbred line, 7927-4-309, from USDA-ARS germplasm (Lewellen) were selected as tolerant controls.

Sugar beet genotypes were grown in the greenhouse. Before planting, seeds were surface-disinfected by soaking in 0.5% sodium hypochlorite for 20 min, rinsed with sterile water and incubated overnight (with shaking at 150 rpm) with 50 mL of 0.3 % hydrogen peroxide to obtain a greater homogeneity in plant germination (McGrath 2000). Metalaxyl was applied as a fungicide to control damping-off by *Pythium* species. The seeds were planted in the greenhouse (16 hr light cycle, 24 °C), in peat-based potting mix (Sure-Mix, Michigan Grower Products, Galesburg, MI). Ten days after planting, for each variety, three seedlings per pot were transplanted to twelve plastic pots to ensure a uniform plant stand. After transplanting a slow-release fertilizer

(Osmocote 14-14-14, Everris International, Netherlands) was added to each pot to fertilize plants.

Isolates and inoculum preparation

Two virulent isolates, F19 and Fob220a, of *Fusarium oxysporum* f. sp. *betae* were used as inoculum. F19 and Fob220a (Hanson et al. 2009), belong to different genetic sub-groups (Hill et al. 2011). Both isolates were maintained desiccated on sterile glass fiber filter paper at -20°C for long term storage (Hanson and Hill 2004), and placed on potato dextrose agar (PDA) for a week before use. A plug 5 mm of hyphae from the actively growing edge of a colony on PDA was transferred to a flask containing V8 juice media. Flasks were incubated for 7 days in an incubator shaker (at 27 °C and 150 rpm).

At the end of the incubation, the conidia were counted using a hemocytometer and the concentration was adjusted to 4×10^4 conidia per ml with sterile water. Five-week-old sugar beet plants were inoculated using a root-dip inoculation method (Hanson and Hill 2004). For each variety, the plants were removed from the substrate and the roots were washed with tap water. Twelve plants per treatments were randomly chosen and inoculated by dipping the roots for 8 min in the spore suspension of one isolate, shaking every minute. Mock inoculation was performed using sterile water. Plants were transplanted to the pots with 3 plants per pot. Plants were watered when soil was dry on the surface. A week after inoculation, damaged and dead leaves were removed to avoid subsequent confusion of disease ratings with transplanting damage.

***Fusarium* symptoms rating**

Individual plants were rated weekly from 2- to 6-weeks post inoculation. Foliar Fusarium yellows symptoms were scored using a modified 0-5 rating scale (Hanson and Hill, 2004), where 0 = healthy plants, 1= leaves wilted or with small chlorotic areas, 2= leaves with inter-venial chlorosis, with entire leaves chlorotic, 3= leaves showing necrotic spots and dying but with less than half leaves per plant affected, 4= half or more than half the leaves dead, 5= entire plant dead. After 6 weeks, plants were harvested and the roots were cut open and examined for rot and vascular discoloration symptoms. For each variety, two randomly chosen roots for each treatment were surface-disinfected in 0.5% sodium hypochlorite for 30 sec and placed

onto PDA plates to confirm the presence of *Fusarium* isolates. The area under the disease progress curve (AUDPC), which also considers the time course of symptom development, was calculated for the 6 weeks for each plant and the mean AUDPC determined for each isolate or control. A disease severity index (DSI) was calculated using the following formula: DSI= [∑Ratings for each plant / (5 x Number of plant rated)] x 100, where 5 is the highest disease rating. The DSI ranged from 0 = all plants healthy to 100 = all rated plants dead. Experiments were done twice.

Data analysis

Data were subjected to analysis of variance (ANOVA) and the Tukey HSD Test was applied for genotype means comparison at P< 0.05. The package used for statistical analysis was Statistica 12.0 (StatSoft Inc. Tulsa, OK, USA).

RESULTS

The 29 inoculated sugar beet lines differed significantly (P<0.05) for their response to the two fungal isolates (Table 1).

Table 1: Average area under the disease progress curve (AUDPC) values for Fusarium oxysporum *f. sp.* betae *isolates F19 and Fob220a causing Fusarium yellows and root rot on sugar beet. Results show the average values for 29 sugar beet lines. Sterile water was used as control.*

Treatment	AUDPC Mean	
F19	102,43	a
Fob220a	82,72	b
Steril water	14,49	c

Means with different letters within columns differ by Tukey's post hoc test at p = 0.05.

The two tested isolates were both virulent, but they caused significantly different disease severity values on the sugar beet lines ($p<0.05$). Isolate F19 caused greater disease symptoms than Fob220a (Table 1), except for the lines 20, 309, A3 and 2 where the AUDPC was higher with the Fob220a inoculum (Table 2).

The highest AUDPC value was recorded for the susceptible genotypes 9, followed by lines 12, 6 and 1 inoculated with F19. The genotypes inoculated with Fob220a showing the highest AUDPC values were 6 and 12. Sugar beet lines 7927-4-309 named 309, A2, A3, A4 and A7 produced lower AUDPCs when inoculated with both isolates, while line 3 inoculated with Fob220a showed lower AUDPC than when inoculated with F19. Line 20 showed lower AUDPC with F19 compared to Fob220a (Table 2).

Table 2: Average area under the disease progress curve (AUDPC) value for 24 plants (12 per treatment, replicated twice) of sugar beet lines screened for tolerance to two Fusarium oxysporum *f. sp.* betae (*FOB) isolates F19 and Fob220a. Varieties "20" and* 7927-4-309, named 309 *were resistant controls*.

	F19			Fob22a	
Sugar beet line	AUDPC		Sugar beet line	AUDPC	
9	141.30	a	6	119.58	a
12	140.30	ab	12	119.44	a
6	134.80	abc	1	116.67	a
1	129.40	abcd	2	107.04	ab
10	114.20	abcde	17	103.10	abc
5	110.70	abcde	5	97.85	abc
7	109.50	abcde	13	88.52	abcd
17	105.00	abcdef	10	85.46	abcd
15	104.90	abcdef	7	84.15	abcde
2	104.90	abcdef	9	82.40	abcde
A9	101.10	abcdefg	15	80.50	abcde
18	99.02	abcdefg	8	79.33	abcde
11	98.73	abcdefg	18	76.56	abcde
13	97.71	abcdefg	A9	71.52	abcdef
14	96.69	abcdefg	4	71.17	bcdef
8	89.10	bcdefgh	20	63.73	bcdefg
4	88.38	cdefgh	14	62.56	bcdefg
19	83.27	cdefgh	11	61.40	bcdefg
A6	81.81	defgh	A5	59.50	bcdefg
3	78.90	defgh	16	58.48	bcdefg
A5	78.75	defgh	19	58.04	bcdefg
A8	77.15	efgh	A6	57.17	cdefg
16	72.33	efgh	A8	56.58	cdefg
A7	72.04	efgh	A3	44.19	defg
A4	54.10	fghi	A7	37.63	defg
20	49.73	ghi	A4	32.08	efg
A3	42.29	hi	3	31.21	efg
A2	17.21	i	309	23.04	fg
309	12.25	i	A2	14.44	g

AUDPC was calculated from scores evaluated every week for 6 weeks using a 0 to 5 rating scale. Numbers represent mean AUDPC. Means with different letters within columns differ by by Tukey's post hoc test at p = 0.05.

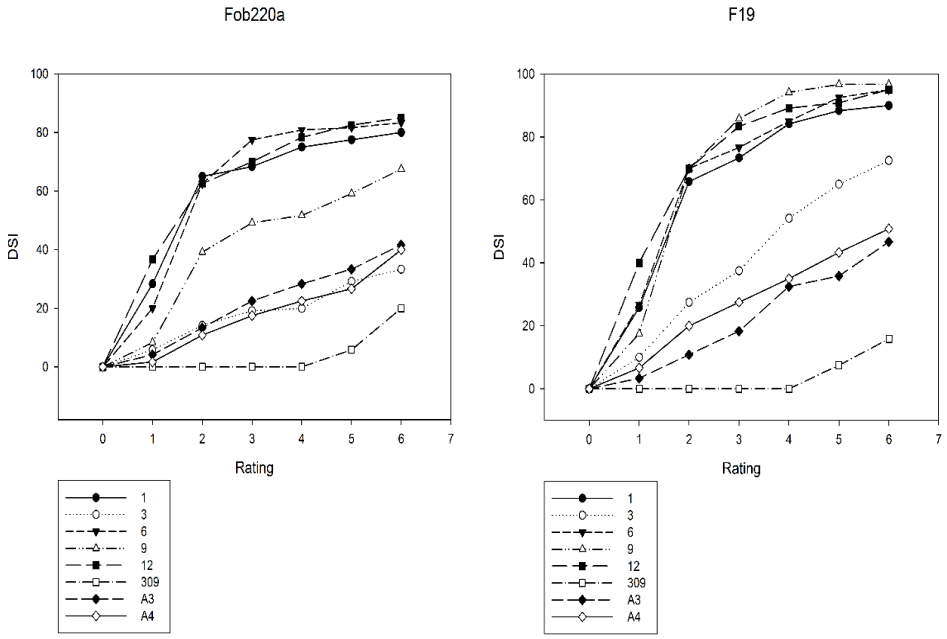

Fig. 1: Disease severity index (DSI) of four high susceptible sugar beet lines: 1, 6, 9 and 12 and four highly tolerant lines: 3, 7927-4-309 (*309), A3 and A4*.

The disease severity in susceptible lines increased rapidly from the first rating, while the more tolerant lines showed a slow progress in disease severity (Fig. 1).

The severity of infection was very high in the susceptible lines with a rapid development of disease. In these genotypes typical interveinal chlorosis appeared at two weeks post inoculation (wpi). Subsequently, the leaves began to die and several plants were killed by three wpi. The first symptoms usually arose one or two weeks later in the tolerant lines than in the susceptible plants.

At the end of the rating the total number of dead plants inoculated with F19 and Fob220a were 48% and 27%, respectively. Lines 12, 6 and 9 showed more than of 80% of dead plants when inoculated with F19, with line 9 in particular reaching more than 96% mortality at the end of the experiment. In contrast, the control 309 line showed no dead plants with either FOB isolate, as did line A2 with F19 and A4 with Fob220a. Lines 3, A2, A6, A7, 19, A5, A8, 4, all inoculated with Fob220a showed less than 10% of dead plants. Line 20 showed the same mortality with both isolates (Fig. 2).

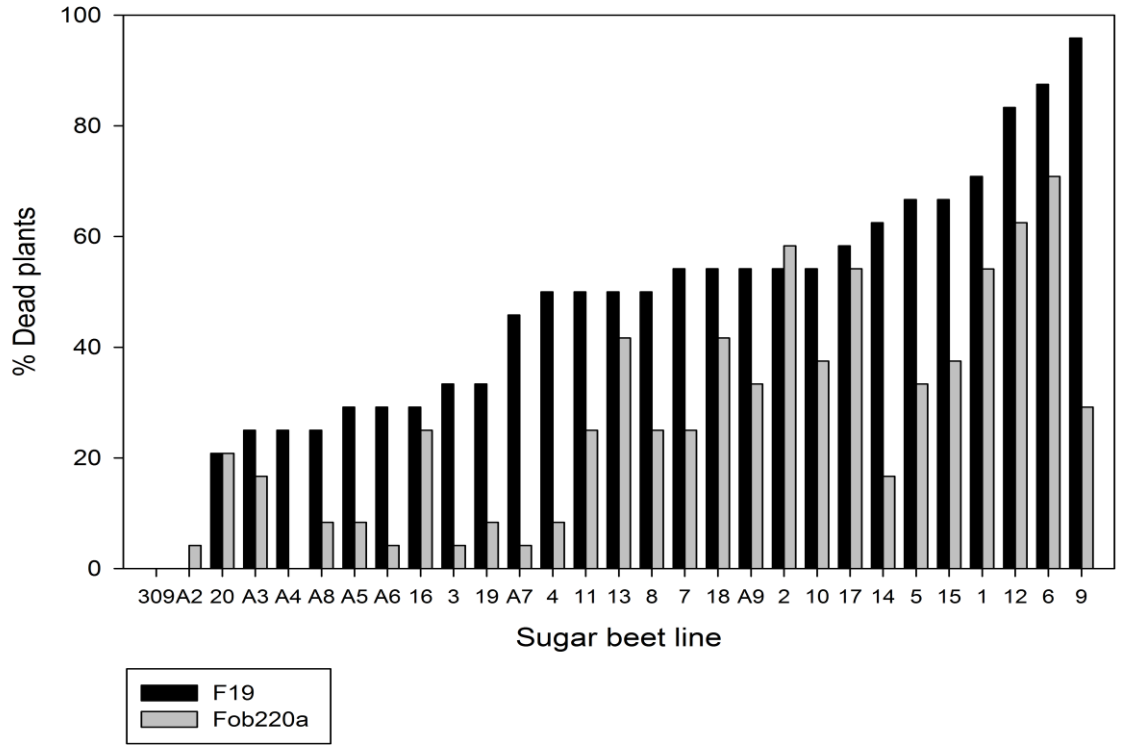

Fig. 2: Percentage of dead plants for each of the 29 tested sugar beet lines treated with two different isolates of Fusarium oxysporum *f.sp.* betae *(F19 and Fob220a) at the end of the experiments, seven weeks after inoculation.*

The correlation between AUDPC and dead plants was 0.87 and highly significant ($P<0.05$). The control plants inoculated with sterile water did not exhibit symptoms of infection by *F. oxysporum* during the experiment.

At the end of the experiment all plants were harvested and roots were cut open and visually compared for root symptoms. All non-inoculated control plants presented healthy roots, while in the plants inoculated with Fob220a and F19 both discoloration and root rot were visible. The internal discoloration varied according to the genotype, and in the susceptible lines black-brown external discoloration was observed. Moreover, *Fusarium* was isolated from the roots of all lines inoculated under greenhouse condition and was confirmed as *Fusarium oxysporum* f. sp. *betae,* morphologically similar to the isolates used in inoculations. No *Fusarium* was isolated from non-inoculated control plants.

DISCUSSION

Fusarium-induced diseases are a widespread threat to sugar beet production (Panella and Lewellen 2005). The use of resistant cultivars is the most cost-effective and sustainable management strategy (Fritsche-Neto and Borém 2012). The purpose of our study was to evaluate the responses of sugar beet germplasm lines to *Fusarium oxysporum* f. sp. *betae* in order to identify potential sources of resistance that could serve as valuable genetic resources for disease resistance breeding programs. The response of a collection of sugar beet germplasm was evaluated against two isolates of *Fusarium oxysporum* under greenhouse conditions and the cumulative progress of disease was estimated using AUDPC.

Both isolates used in this screening were highly virulent as reported in other studies (Hanson et al. 2009, Hill et al. 2011) and F19 caused more severe disease symptoms than Fob220a, as previously demonstrated by Hanson et al. (2009). The tolerant and susceptible lines showed the same infection levels with both isolates. These two tested isolates were reported as *Fusarium oxysporum* f. s. *betae* that cause Fusarium yellowing with vascular discoloration in affected sugar beets (Hanson et al. 2009).

In this study, surprisingly, in the susceptible genotypes we observed also root rot, the typical symptom caused by *F. oxysporum* forma specialis *radicis-betae* (Harveson and Rush 1998, Harveson 2009). To our knowledge, this is the first report of root rot symptoms on sugar beet plants inoculated with *Fusarium oxysporum* f. s. *betae*. This is probably due to a germplasm effect, so further tests are needed to clarify why this unusual response is present in the Italian sugar beet lines and if the root rot is due only to the fungal strains used in this study or whether there is also an host component.

A wide variability was observed in the tested sugar beet lines and AUDPC values varied greatly between susceptible and tolerant lines, with susceptible lines showing higher AUDPC values than the tolerant lines, thus indicating a reduced disease progress in tolerant genotypes. The significant differences among the tested lines could be ascribed to the presence in the more tolerant lines of resistance genes to *Fusarium* infection. However, tolerance observed in greenhouse conditions should be tested in the field under natural *F. oxysporum* f.sp. *betae* infection (Hanson et al. 2009) especially as variability has been reported with different pathogen isolates (Ruppel 1991,Hanson et al. 2009) and for different geographic regions. It is even possible that

the lines identified in the present work as moderate resistant may show more resistant under natural infected field if there are not optimal disease conditions.

Genetic variability is essential to develop disease resistant plants (Michelmore et al. 2013) and by means of this study new resistant lines were identified that may be used for a breeding program as donors of *Fusarium* resistance and combined with high-yield genotypes in order to improve food security.

The interaction between *Fusarium oxysporum* and host plants has been well studied in several crops and resistance genes have been identified (Diener and Ausubel 2005, Oumouloud et al. 2008, Sharma and Muehlbauer 2007, Sela-Buurlage et al. 2001). Moreover, in tomato and melon the genes involved in *Fusarium* resistance are dominant (Sela-Buurlage et al. 2001, Joobeur at al. 2004). The genetic control for resistance to *Fusarium*–induced diseases in sugar beet is still unknown. Fewer evidences are available about *F. oxysporum*-resistance sources in sugar beet with respect to other crops. Therefore, more research is needed to evaluate whether the genetic control is monogenic or polygenic. The monogenic resistance, also called qualitative resistance, is easily selected in a breeding program (Boyd et al. 2013) whereas the other category of plant disease resistance, known as quantitative disease resistance (QDR), is conferred by multiple genes with more durable resistance (Zhang et al. 2013).

The next step will be to identify molecular markers linked to root rot tolerance to use in marker-assisted breeding. To achieve this goal two possible approaches can be applied: bulk segregant analysis (BSA) (Michelmore et al. 1991) and association analysis (Cardon and Bell 2001). BSA led to successful identification of markers linked to rhizomania and nematode resistance in sugar beet (Barzen et al. 1997, Stevanato et al. 2014), and offers promise for *Fusarium* resistance. To use this method it is necessary to develop a population resulting from a cross between a highly resistant and a highly susceptible line, such as some of those identified in this work, which segregates for the *Fusarium* resistant trait. An association study using these tested lines may be a useful tool to identify polymorphisms associated with the *Fusarium oxysporum* tolerance as previously reported for *Physalis peruviana* interaction with *F. oxysporum* (Enciso-Rodríguez et al. 2012).

In conclusion, this first screening for *Fusarium oxysporum* tolerance on an Italian germplasm set led to identification of genotypes highly tolerant to Fusarium. These lines could possess resistant genes that could be transferred by a breeding program to high yield lines to increase sugar beet resistance to *Fusarium.*

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CONTRIBUTE 3

Host-pathogen interactions in sugar beet lines affected by *Fusarium oxysporum* f.sp. *betae*

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ABSTRACT

The soil-borne fungus *Fusarium oxysporum* may cause both Fusarium yellows and Fusarium root rot diseases with severe yield losses in cultivated sugar beet worldwide. These two diseases, causing the same foliar symptoms but different root response, are due to two distinct *Fusarium oxysporum* formae speciales. Fusarium yellows induced by the *Fusarium oxysporum* f.sp. *betae* presents vascular discoloration, whereas Fusarium root rot due to the presence of the *F. oxysporum* f.sp. *radicis-betae* is characterized by black rot in the root. The aim of this work was to study the host-pathogen interaction of sugar beet lines from two different sugar beet breeding programs with isolates of *Fusarium oxysporum* f. sp. *betae*. Eight susceptible sugar beet lines, selected by USDA-ARS (US) and UNIPD (University of Padova, Italy), were inoculated with three different isolates of *Fusarium oxysporum* f. sp. *betae*, the causal agent of Fusarium yellows. All inoculated lines developed disease symptoms, but disease severity (AUDPC) differed significantly ($P<0.05$) among lines. Two lines from UNIPD, 6 and 9, were the most susceptible to the disease, whereas the other lines showed similar levels of disease. Isolates of *F. oxysporum* f. sp. *betae* from different genetic groups, differ significantly ($P<0.05$) in pathogenicity. Five weeks after inoculation the plants were harvested and the root examined. Severe root rot has been observed in the susceptible UNIPD lines inoculated with isolates that had never shown root rot in the USDA germplasm. Our results show that Fusarium root rot is induced not only from different *Fusarium oxysporum* isolates that infect plants, but also is due to different host factors.

KEYWORDS: sugar beet, root rot, host-pathogen interaction, germplasm

INTRODUCTION

Fusarium oxysporum Schelecht. is a ubiquitous soil-borne fungus that includes pathogenic and non- pathogenic strains (Appel and Gordon 1996). Pathogens can induce vascular wilt or root rot on a wide range of plant, causing severe crop losses (Olivain and Alabouvette 1999, Kroes et al. 1998). *F. oxysporum* is considered to be a hemibiotroph (Thaler et al. 2004) because the initial stages of interaction by this pathogen are biotrophic, but later stages are comparable to the lifestyle of necrotrophic pathogens (Kidd et al. 2011). Pathogenic strains have a high level of host specificity and isolates have been divided into more than 120 different *formae speciales* (f. sp.) based on the host or group of hosts they attack (Armstrong and Armstrong, 1981). A particular *forma specialis* can be subdivided into physiological races based on their virulence to a set of differential host cultivar (Correll 1991).

Sugar beet (*Beta vulgaris* L.) is grown in all the temperate zones and the crop contributes at least 20% of the world's sugar supply (FAO STAT 2014). One of the main pressures to sugar beet cultivation is damage caused by pathogens, including viruses, nematodes, and fungi, which cause severe yield losses (Biancardi et al. 2010). The soil-borne *Fusarium oxysporum* has the capacity to infect sugar beet and may cause severe losses in production in cultivated sugar beet worldwide and the fungus can cause either Fusarium yellows or Fusarium root rot (Harveson 2009). *Fusarium oxysporum* penetrates the roots and grows into the vascular system, producing toxic elements that lead to functional collapse, wilting, yellowing and often the death of the infected plant (Harveson 2009). Fusarium yellows was first reported in Colorado by Stewart in 1931. Affected sugar beets present interveinal yellowing on the older foliage. Frequently, only one side of the leaf is affected first. Root symptoms are characterized by vascular discoloration (Stewart 1931). In 1989 a disease similar to Fusarium yellows, but with a severe root rot never observed before was discovered in Texas and the isolate of *F. oxysporum* that caused that root rot was proposed to be a new forma specialis (Martyn et al. 1989). The two different formae specialis (f. sp.) that cause Fusarium disease on sugar beet are: *Fusarium oxysporum* f.sp. *betae* that causes Fusarium yellows and *F. oxysporum* f.sp. *radicis-betae* which can lead to Fusarium root rot (Hanson and Jacobsen 2009, Harveson 2009).

Recently some European sugar beet varieties showed root rot associated with *Fusarium* infection in the field. When these varieties were tested with US isolates of *Fusarium oxysporum*, classified as *F. oxysporum* f.sp. *betae* (yellows type), a root rot

was observed. In the present work, we evaluated the reaction of two genetically different sugar beet collections to distinct isolates of *F. oxysporum* f. sp. *betae* from two of the three *F. oxysporum* genetic groups (Hill et al. 2011) with the aim of investigating this response and test if it is due to an isolate effect or there is an effect of the beet germplasm. We compared the response of four US susceptible sugar beet germplasm lines and a collection of four susceptible sugar beet lines from University of Padova (Italy).

MATERIALS AND METHODS

Plant materials

Eight susceptible sugar beet lines were examined. Four Italian sugar beet lines from DAFNAE- Department of Agronomy, Food, Natural resources, Animals and Environment (University of Padova, Italy), 6, 7, 9 and 18, and four USDA-ARS varieties, FC716 (Panella et al. 1995), C869 (Lewellen 2004), EL51 (Halloin et al. 2000) and SP7322 (Coe and Hogaboam 1971) were grown in the greenhouse.

Seed was soaked in 0.5% sodium hypochlorite for 20 min, rinsed with water, and incubated overnight (with shaking at 150 rpm) in 50 mL of 0.3 % hydrogen peroxide to promote uniformity in germination (McGrath, 2000). Metalaxyl was used to control damping-off by *Pythium* species. Seed was planted in peat-based potting mix (Sure-Mix, Michigan Grower Products, Galesburg, MI) in plastic pots. Ten days after planting, three seedlings per pot from each variety were transplanted to twelve plastic pots, to obtain uniform plants. Plants were fertilized with slow-release fertilizer (Osmocote 14-14-14, Everris International, Netherlands) added to each pot after transplanting. The plants were grown in the greenhouse (16 hr light cycle, 24 °C).

Isolates and inoculum preparation

We used 3 *Fusarium oxysporum* f. sp. *betae* isolates, Fob220a, Fob13 and Fob216c, representing two of the three known genetic groups (Hanson et al. 2009, Hill et al. 2011). Fusarium inoculum was prepared by taking a plug of hyphae from an actively growing edge of a colony on the potato dextrose agar (PDA) plate, which was then

transferred to a flask containing V8 agar media. Flasks were incubated for 7 days in an incubator shaker (150 rpm, 27 °C).

Conidia concentration was estimated using a hemocytomer and adjusted to 4 x 10^4 conidia per ml with sterile water. Five-weeks-old plants were inoculated using the root-dip inoculation method (Hanson and Hill 2004). For each variety, the plants were gently removed from the soil and the roots were rinsed with tap water. Twelve plants per treatments were randomly chosen and their roots were submerged in the Fusarium spore suspension for 8 min with the suspension shaken every minute. Sterile water was used as control. Plants were replanted in pots with 3 plants/ pot. Plants were watered when soil was dry on the surface. A week after inoculation, damaged and dead leaves were removed to avoid confusion between transplanting and fungus damage.

Fusarium Rating

The response to *F. oxysporum* isolates was evaluated under controlled environment conditions. Beginning two weeks after inoculation, individual plants were rated weekly for foliar Fusarium yellows symptoms for 4 weeks using a modified 0-5 rating scale (Hanson and Hill, 2004), where 0 = healthy plants, 1= leaves may be wilted or with small chlorotic areas, 2= leaves with intervenial chlorosis, with entire leaves chlorotic, 3= leaves showing nectotic spots, or nectrotic and dying but only less than half leaves are affected, 4= half or more of the leaves dead, 5= entire plant dead.

Root evaluation

Five weeks after inoculation, entire plants were de-potted and washed under running tap water to remove the attached soil. The roots were cut open longitudinally to examine root symptoms. For each variety, two randomly chosen roots were placed onto PDA plates, after surface disinfection in 0.5% sodium hypochlorite for 30 sec, to confirm the presence of *Fusarium* isolates.

Statistical method

The area under the disease progress curve (AUDPC) was calculated for the 4 week ratings for each plant and the mean AUDPC determined for each isolate. Experiments were done twice.

AUDPC was subjected to analysis of variance (ANOVA) and comparison of genotype means was done with Tukey's HSD test P=0.05. The package used for analysis was Statistica 12.0 (StatSoft Inc. Tulsa, OK, USA).

RESULTS

Virulence of isolates

All 8 sugar beet lines examined in the greenhouse were susceptible to Fusarium. The intensity of foliar disease symptoms differed significantly (P< 0.05) for their response to Fusarium: lines 6 and 9 from UNIPD were significantly more affected then the other lines (Table 1).

Table 1: Average area under the disease progress curve (AUDPC) value for 24 plants (12 plants per treatment, replicated twice) for sugar beet lines screened with three Fusarium oxysporum *f. sp.* betae (*FOB) isolates Fob220a, Fob216c and Fob13.*

Line	Origin	AUDPC	
6	Italy	39.38	a
9	Italy	33.64	a
7	Italy	19.83	b
18	Italy	15.56	b
SP7322	US	12.06	b
FC716	US	11.86	b
EL51	US	10.40	b
C869	US	8.26	b

Different letters denote significant differences at p<0.05 using a Tukey's post hoc test.

All plants inoculated with sterile water remained symptomless. All three isolates of *F. oxysporum* f.sp. *betae* used in the greenhouse experiment, Fob220a, Fob216c and Fob13 were pathogenic and caused foliar disease symptoms in all tested genotypes.

Depending on the variety, symptoms started to appear 3 weeks after inoculation and they increased at different levels. Isolate Fob220a was more virulent than the other two isolates for all the inoculated genotypes, whereas Fob13 caused fewer disease symptoms (Table 2). Fob216 had the same level of virulence in all the tested genotypes (Table 3).

Table 2: Average area under the disease progress curve (AUDPC) values for Fusarium oxysporum *f. sp.* betae *isolates Fob220a, Fob216c and Fob13 causing Fusarium damages on sugar beet. Sterile water was used as control. Results show the average values for 8 sugar beet lines.*

Teatment	AUDPC	
Fob220a	23.77	a
Fob216c	18.74	ab
Fob13	14.11	b
Sterile water	1.24	c

Different letters denote significant differences at p<0.05 using a Tukey's post hoc test.

The highest AUDPC mean values occurred on the cultivar 6, followed by the cultivar 9 for the isolates Fob220a and Fob13 (Table 3). The lowest AUDPC mean value was recorded on the cultivar EL51 for each isolate (Table 3).

Two plants from different germplasm, SP7322 and 7, both inoculated with isolate Fob216c, died two weeks after inoculation and the plant from genotype 7 showed complete rot root. Only one plant (from genotype 6) died using isolate Fob13. Isolate Fob220a killed five and six plants of the genotype 6 and 9, respectively. One plant each of FC716 and SP7322 also was killed by the isolate Fob220a.

Table 3: Response of eight sugar beet lines to three Fusarium oxysporum *f. sp.* betae *isolates: Fob220a, Fob216c and Fob13. Values are the average area under the disease progress curve (AUDPC) for 24 plants (12 plants per treatment, replicated twice) rated weekly for four weeks using a 0-5 rating disease scale.*

		Fob13		Fob216c		Fob220a	
Line	Origin	AUDPC		AUDPC		AUDPC	
6	Italy	28.88	a	32.67	a	56.58	a
9	Italy	27.13	ab	23.33	a	50.46	a
18	Italy	13.71	bc	19.25	a	13.71	b
7	Italy	12.25	c	32.96	a	14.29	b
SP7322	US	8.75	c	17.50	a	9.92	b
FC716	US	8.17	c	8.46	a	18.96	b
C869	US	7.29	c	7.58	a	9.92	b
EL51	US	6.71	c	8.17	a	16.33	b

Values for an isolate followed by the same letter are not significantly different by Tukey's HSD test at P<0.05.

Root infection

Five weeks after inoculation, all plants were harvested and roots were cut open longitudinally and visually compared for root symptoms. Plants of the un-inoculated treatments showed healthy roots, whereas plants inoculated with the three FOB isolates with foliar symptoms showed different root symptoms as vascular discoloration or root rot.

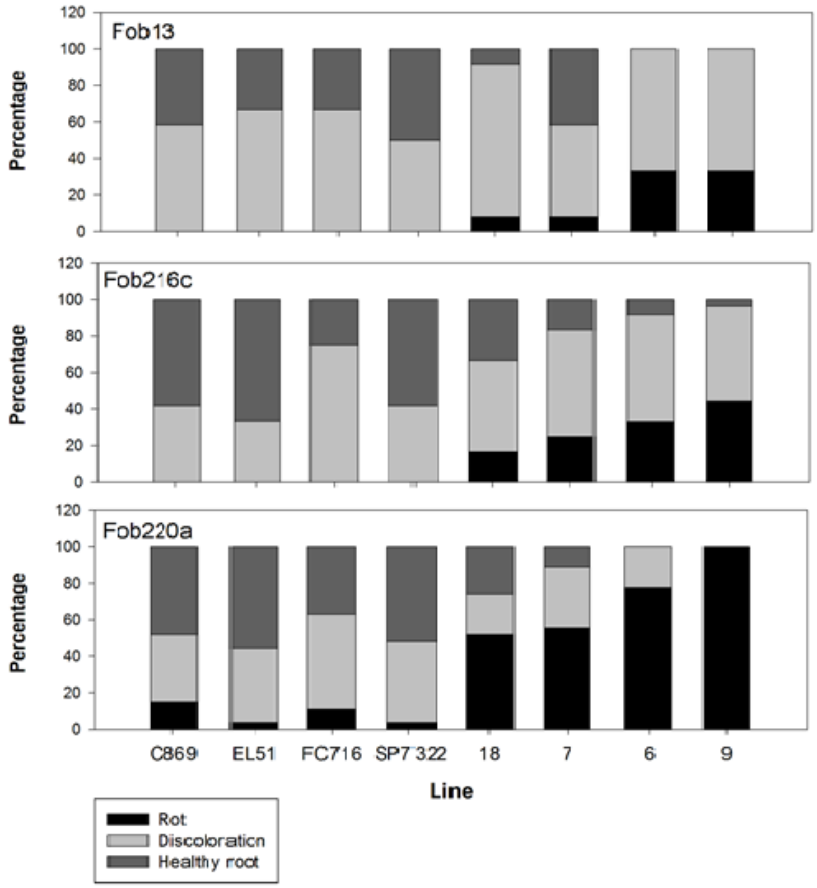

Figure 1: Root symptoms of eight sugar beet lines to three Fusarium oxysporum *f. sp.* betae *isolates: Fob220a, Fob216c and Fob13. Values are the percentage of rot, discoloration and healthy roots for 24 plants (12 plants per treatment, replicated twice) at five weeks after inoculation.*

Internal discolored lesions occurred on taproots of the plants from USDA and UNIPD lines (Figure 1). External symptoms were observed in all Italian accessions inoculated with each isolate. In the most severe host-pathogen interaction, roots of Italian lines were necrotic and visually destroyed. Disease symptoms were always more severe in inoculation with the isolate Fob220a. Lines 6 and 9 inoculated with Fob13 and Fob220a did not have healthy roots, and 100% of plants of line 9 inoculated with Fob200a showed rot symptoms. Isolates Fob13 and Fob216c did not cause rot in all the tested US genotypes, whereas isolate Fob220a caused rot in more than 50% of UNIPD plants and less than 10% in the USDA plants.

All isolates were re-isolated from roots tissue samples of each sugar beet lines after the harvest and the Fusarium isolates matched the species used in inoculations.

DISCUSSION

Fusarium diseases were first reported in US sugar beet fields (Stewart 1931, Martyn et al. 1989), and nowadays they are an emerging problem in the other sugar beet growing areas (Biancardi 2010). In this study, we provide a comparison of two different sugar beet germplam types for the phenotypic reaction to infection by *F. oxysporum* f. sp. *betae*. All the sugar beet lines were known to be susceptible to Fusarium. The purpose of examining the reaction of different sugar beet germplasms to different *F. oxysporum* f. sp. *betae* is to understand if there is a different response linked to the host component and not only due to a strain component.

The three isolates used in this study were previously described to be *Fusarium oxysporum* f. sp. *betae*, the causal agent of Fusarium yellows on sugar beet, that cause a series of leaves symptoms as wilting and necrosis that can lead to plant death and vascular browning of the root but not root rot (Hanson et al. 2009). Susceptibility to *Fob* among sugar beet lines was assessed by development of foliar disease symptoms and root symptoms at the end of the rating.

The evaluation of leaf symptoms allowed us to observe that all the tested isolates were pathogenic and they caused significantly different disease severity as has been showed in other studies (Hanson and Hill 2004, Hanson et al. 2009). Fob220a was the most virulent isolates but the lines 18, 7, and SP7322 showed a high AUDPC with Fob216c, and a lower one with the other two isolates. All the tested sugar beet lines developed Fusarium symptoms, but they was more severe in the Italian lines 6 and 9.

The visual examination of root symptoms was performed on 10-weeks-old plants at 5 weeks after inoculation and permitted a clear discrimination between susceptible and healthy plants and was able to identify the different susceptible responses.

The three *Fob* used in this work showed ,as previously reported, vascular discoloration in sugar beet (Hanson et al. 2009). In fact, all the tested US lines and Italian plants of lines 7 and 18 developed symptoms of wilting and yellowing with the same intensity, but after the harvest, when the roots had been analyzed, we observed discoloration for all the lines, and rot was present in the Italian roots. Rot usually started from the root tip and developed a black discoloration in infected root parts. In the US lines, no rot root was observed in plants inoculated with isolates Fob13 and Fob216c, while lines treated with Fob220a showed some plants with internal discoloration that spread to a rot near the vascular discoloration areas.

This screening of *F. oxysporum* f. sp. *betae* on different sugar beet germplasms suggested that there is a different range of responses to the pathogen. This study showed that sugar beet lines vary in their responses to infection by different isolates as reported previously (Hanson et al. 2009). Our work suggests that the differences in disease symptoms of lines caused by *F. oxysporum* in relation to the severity of disease also depend on the variety and the relative genotype susceptibility of these isolates.

An option for screening different germplasms could be to use a mixing of different isolates for aggressiveness and origin to evaluate the variation between susceptible lines from different germplasms. However, in this study, we observed the response of susceptible sugar beet lines from different germplasm using three different pathogenic isolates, and these isolates clearly showed the different root responses on the lines tested.

Plants have evolved complex defense mechanisms during the co-evolution of plants and pathogens (Dangl and Jones 2001) and, although all varieties could be considered susceptible, there are differences in disease reaction that may be due to genetic variation within the sugar beet germplasms. Because of the Fusarium diseases were a serious threat to the US sugar beet production, probably even the susceptible varieties present some genetic component able to reduce the Fusarium infection due to the unconscious selection of defense against a natural enemy. On the other side, the Fusarium diseases were not a problem in the past in Europe and the germplasm had never co-existed with highly virulent isolates, and therefore the susceptible lines did not present any defense mechanism against this pathogen. More studies are needed to characterize any possible genetic link between breeding selection and resistance to *Fusarium oxysporum* f. sp. *betae*.

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CONTRIBUTE 4

Identification of SNP markers associated to *Fusarium* resistance in sugar beet

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ABSTRACT

Fusarium spp. cause severe damage in many agricultural crops including sugar beet. Sugar beet needs to be protected from these soil-borne pathogens to guarantee an optimal sugar yield in the field. The genetic control is the key to overcoming this disease. Identification of single nucleotide polymorphism (SNPs) markers linked to the resistance can be a powerful tool for the introgression of valuable genes needed to develop *Fusarium*-resistant varieties. A candidate gene approach was carried out to identify SNP markers linked to *Fusarium* resistance in sugar beet. Five resistant analogue genes (RGAs) were screened by means of HRM analysis in a set of resistant and susceptible lines to *Fusarium*. HRM polymorphisms were observed in 80% of amplicons. Two polymorphisms resulted associated with Fusarium resistance ($P<0.05$). The amplicons that showed association were sequenced and two SNPs were identified. The association was further validated on 96 susceptible and 96 resistant plants using a competitive allele-specific PCR (KASPar) technology. The selected SNPs could be used for marker-assisted breeding of *Fusarium* resistance in sugar beet.

KEYWORDS: sugar beet, Fusarium resistance, HRM analysis, KASPar assay

INTRODUCTION

Sugar beet (*Beta vulgaris* L.) crop is grown in all the temperate areas and produces up to 20% of the world's sugar supply (FAO 2013). Its production is challenged by several endemic and emerging diseases. Among fungal diseases, the soil-borne fungus *Fusarium oxysporum* can cause two diseases known as Fusarium yellows and Fusarium root rot and affecting sugar beet yield in US, Russia, Ukraine and China (Harveson 2009). Symptoms of *Fusarium* include foliar wilting and interveinal yellowing with vascular discoloration in case of Fusarium yellows and a black root rot in Fusarium root rot plants (Stewart 1931, Martyn et al. 1989). Crop rotation may not be always an effective control method because the pathogen can survive for long time in the rotation crops (Harveson 2009). Genetic resistance is the best option for managing the disease.

The identification and use of molecular markers linked to the *Fusarium* resistance could reduce not only the time for field screening but also accelerate the development of *Fusarium* resistant varieties. Among molecular markers, single nucleotide polymorphisms (SNPs), are attractive because they are the most abundant source of variation in genomes and amenable to high-throughput automated analysis (Mammadov et al. 2012).

The candidate gene approach is one of the most used methods to identify associations between polymorphisms within pre-specified genes of interest and phenotypes or disease states (Hu et al. 2008, Thudi et al. 2014, Li et al. 2014, Guo et al. 2014). This method directly tests the effect of genetic variants in a gene that may be involved in the control of investigated trait (Pflieger et al. 2001). The candidate gene approach has proven extremely powerful for the identification of genes controlling disease resistance and for the development of markers highly useful for marker-assisted selection (Gebhardt and Valkonen 2001).

Up to now, no genes and quantitative trait loci (QTLs) have been reported for *Fusarium* resistance in sugar beet. Nevertheless, resistance gene analogs (RGAs) can be key targets to identify genes for *Fusarium* resistance in sugar beet. RGAs are obtained on the basis of the conserved domains and they are characterized by their sequence similarity to plant resistant genes. For these reasons, they are considered optimal candidates for selection of markers associated with pathogen resistance. They can be used to develop molecular markers for resistance traits, because of their putative functions in plant innate immune systems and their high level of polymorphism (Liu et al. 2012). RGAs have been isolated from many crops (Palomino et al. 2006, Wenkai et al. 2006, Pei et al. 2007) and a list of 715 RGAs has been recently reported in sugar beet (Dohm et al. 2014).

Besides applications in mutation screening, high resolution melting (HRM) is a rapid and highly sensitive method (Reed et al. 2007). HRM is a close-tube method that does not require fluorescently labeled probes and previous knowledge of the SNP variation is not required. In this topic, HRM assay allows to efficiently identify differences in sequence composition based on the melting curve shape between the amplicons (Liew et al. 2004). Another flexible method with low error rates is the Next-generation Competitive Allele Specific PCR (KASPar). It is a fluorescence-based simple SNP genotyping method developed by LGC Genomics LTd. (Semagn et al. 2013).

In this study, a candidate gene approach was carried out to identify SNP markers linked to *Fusarium* resistance in sugar beet. The identified SNPs could be used for marker-assisted breeding of *Fusarium* resistance in sugar beet.

MATERIALS AND METHODS

Plant material and phenotyping

The plant material used in this study comprises a set of 27 sugar beet pollinators provided by DAFNAE-Department of Agronomy, Food, Natural resources, Animals and Environment (University of Padova, Italy). A resistant pollinator inbred line, 7927-4-309, from USDA-ARS germplasm (R. Lewellen) and a sugar beet variety, 20, provided by Lion Seeds Ltd (UK) and were used as tolerant controls.

Sugar beet lines were screened for resistance to *Fusarium oxysporum* f. sp. *betae* under greenhouse conditions. Five-week-old sugar beet plants were inoculated using a root-dip inoculation method (Hanson and Hill 2004). For each variety, twelve plants per treatments were randomly chosen and inoculated by dipping the roots for 8 min in the spore suspension, shaking every minute. After inoculation, each plant was evaluated weekly for 6 weeks for severity of foliar symptoms based on a 0-5 rating scale (Hanson and Hill 2004), where 0 = healthy plants, 1= leaves wilted or with small chlorotic areas, 2= leaves with inter-venial chlorosis, with entire leaves chlorotic, 3= leaves showing necrotic spots and dying but with less than half leaves per plant affected, 4= half or more than half the leaves dead, 5= entire plant dead. The experiment was repeated twice. The area under the disease progress curve (AUDPC), which also considers the time course of symptom development, was calculated for the 6 weeks for each plant and the mean AUDPC was determined.

DNA isolation, candidate gene selection and mutation screening by high resolution melting (HRM) analysis

DNA was extracted from 20 mg of leaf tissue using the BioSprint 96 DNA Plant Kit in a BioSprint 96 workstation (Qiagen, Germany) following the manufacturer's instructions. The quality and quantity of extracted DNA were evaluated electrophoretically and spectrophotometrically by microfluidic gel electrophoresis with an Agilent 2200

TapeStation system (Agilent Technologies, USA). The DNA concentration of each sample was adjusted to 20 ng ul^{-1}. To ensure high-precision liquid dispensing the robot QIAgility (Qiagen, Germany) was used in the sample preparation.

Five sugar beet resistant gene analogs (RGAs) identified by Hunger et al. (2003) and even reported in Dohm et al. (2014) were screened by means of HRM analysis (Table 1). Primer pairs were designed using Primer3 software (http://www.sgn.cornell.edu/tools/primer3/). The amplicon produced was 160 bp long.

Table 1: List of selected RGA genes

RGA (Hunger et al. 2003)	RGA (Dohm et al. 2014)	Length (bp)	RGA class	Gene Product
8M01	Bv7_171470_ojty	7828	NLcc	Disease resistance protein At4g27190
AD-c-15c	Bv2_043450_zhxk	6688	CNL	Putative disease resistance protein At3g14460
AB4	Bv9_225140_hpxq	4739	NLcc	Putative disease resistance RPP13-like protein 1
8C05	Bv6_147620_tzmc	5233	RLK	Hypothetical protein
9J14	Bv6_152400_uaqg	4165	RLK	Hypothetical protein

CNL= CC-NBS-LRR coiled-coil (CC), nucleotide-binding site (NBS), leucine rich repeat (LRR); NLcc= NBS-LRR; Receptor-like kinases (RLK)

HRM genotyping was performed in 384-well plates using a QuantStudio TM 12 K Flex Real-Time PCR System (Thermo Fisher Scientific, USA) in a total of 5 µl final volume per well. The HRM mix contained 2.5 µl of MeltDoctor HRM Master Mix, 0.45 µl of both forward and reverse primers and 0.2 µl of nuclease-free water. The thermal profile was: 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s, 60 °C for 1 min. The samples were then melted at a ramp rate of 1 % from 60 to 95 °C. All samples were amplified in duplicate. HRM profiles were analyzed with the QuantStudio TM 12K Flex Real-Time PCR software version 1.2.2 (Thermo Fisher Scientific, USA).

Sequencing PCR amplicons

PCR reaction was performed in 15 µL PCR reactions containing 20 ng genomic DNA, 1x PCR buffer (EuroClone, UK), 0.2 µM of each primers, 1.8 mM $MgCl_2$, 150 µM dNTP and 0.8 U of Euro-Taq DNA polymerase (EuroClone, UK). Thermal cycling (TC-512; Techne inc, US) profiles were as follows: 94°C for 30 s, followed by 35 cycles of 94°C for 20 s, 54°C for 20 s, 72°C for 50 s, followed by a final extension at 72°C for 5 min. The resulting PCR products were analyzed on agarose gel and purified using QIAquick PCR purification kit (Qiagen, USA) and then sequenced by a Sanger sequencing platform at Eurofins MWG Operon (Ebersberg, Germany). The sequence data were analyzed and aligned for SNP discovery using Mutation Surveyor version 5.0 (SoftGenetics, USA).

SNP validation

Candidate SNPs were selected and surrounding sequences of them were sent to KBiosciences UK Ltd for primer design and analysis of the SNPs using the PCR-based KASP™ genotyping assay on resistant and susceptible individuals.

Statistical analysis

Phenotypic data were subjected to analysis of variance (ANOVA) using Statistica 12.0 (StatSoft Inc., USA). The statistical tools for genotype analysis of SNPs were provided by SNPStats (Solé et al. 2006). For each SNP we calculated Hardy-Weinberg equilibrium, allele and genotype distributions, and association tests. Odds ratios (OR) and confidence intervals (CI) for the allelic association analysis were estimated by a unconditional logistic regression analysis testing multiple inheritance models (codominant, dominant, recessive, over-dominant, and log-additive) The best model were identified according to the smallest Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) values. The difference in genotype distribution between resistant and susceptible samples was assessed by the Chi-square test.

RESULTS

Phenotypic analysis

Fusarium symptoms of 29 sugar beet lines were evaluated for 6 weeks after pathogen inoculation. Significant differences in disease symptoms (AUDPC) were observed between lines ($P < 0.05$).

The resistant lines showed disease symptoms two weeks later than the susceptible lines. Several susceptible plants were dead three weeks after inoculation. More than 65% of the inoculated plants of lines 6, 9 and 12 were dead at the end of the rating period.

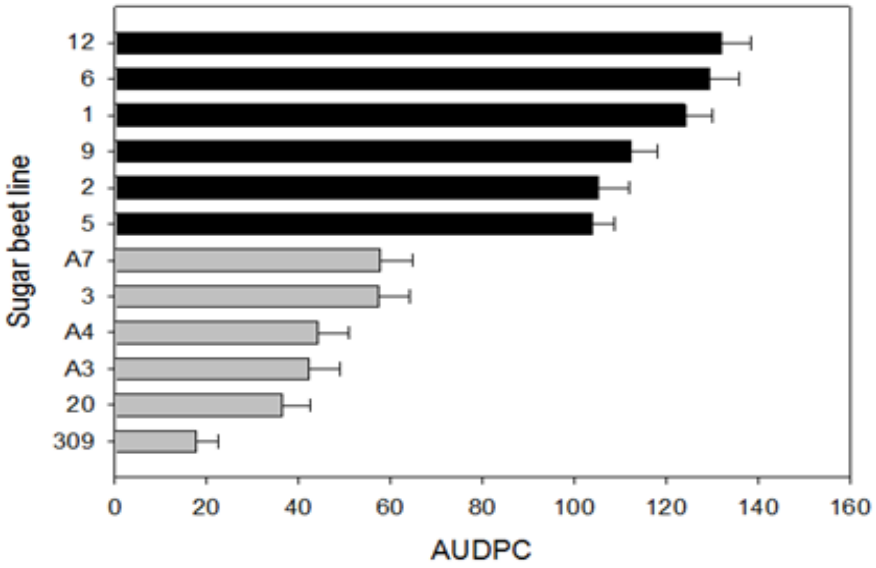


Figure 1: Fusarium symptoms (AUDPC) of the 6 most resistant and the 6 most susceptible sugar beet lines.

Fig. 1 shows the AUDPC values of the 6 most resistant and 6 the most susceptible sugar beet lines selected for high resolution melting (HRM) analysis. The AUDPC average value was 41.71 and 117.78 for the resistant lines and for susceptible lines, respectively.

SNP discovery by high resolution melting (HRM) analysis

Five resistant analogue genes (RGAs) were screened by means of HRM analysis on individual DNA samples of the 6 most resistant and the 6 most susceptible lines. HRM polymorphisms were observed in 80% of amplicons. Two polymorphisms resulted associated with Fusarium resistant ($P<0.05$). The amplicons that showed association were sequenced and two SNPs were identified. The first SNP, located in the exon of Bv2_043450_zhxk gene, was named SNP_Bv2_043450, while the second SNP, located in the exon of the Bv7_171470_ojty gene, was named SNP_Bv7_171470. The AA genotype resulted associated with *Fusarium* resistance both at the SNP_ Bv2_043450 and SNP_Bv7_171470 loci. Fig. 3 shows representative HRM profiles from SNP_Bv2_043450 and SNP_Bv7_171470 amplicons obtained from resistant and susceptible samples.

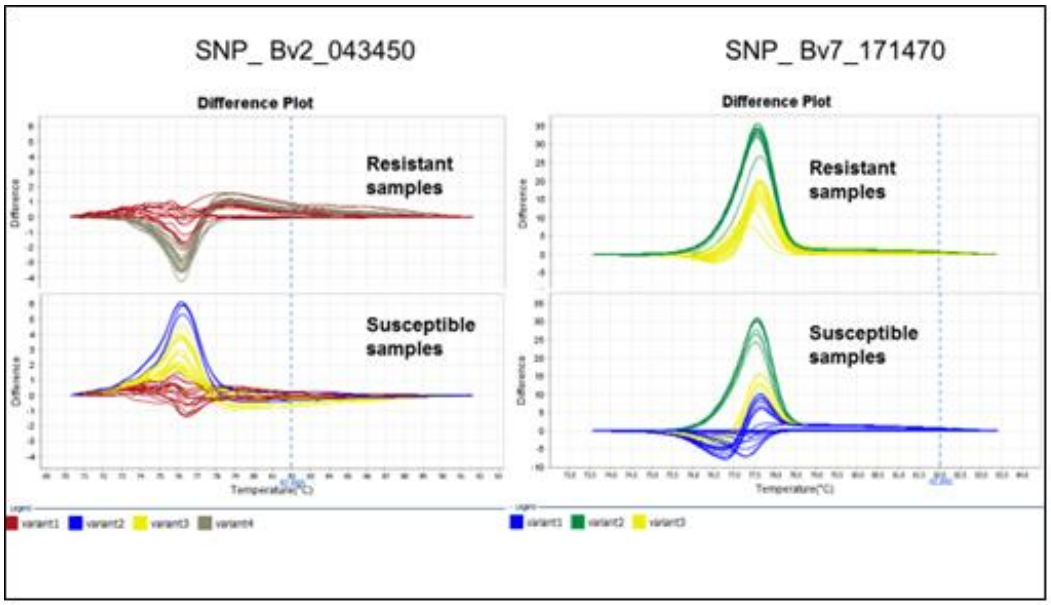
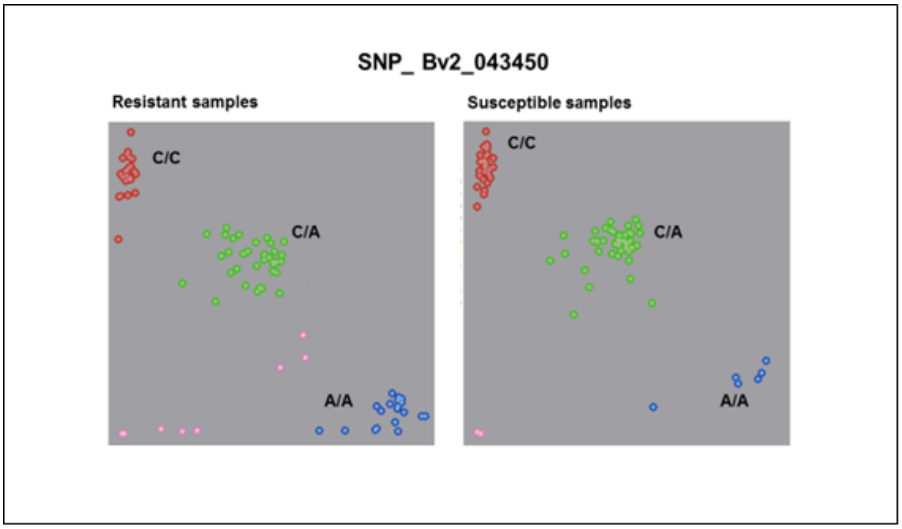


Figure 3: Representative HRM profiles from SNP_ Bv2_043450 and SNP_ Bv7_171470 amplicons.

SNP validation by KASPar genotyping

The two SNPs associated with *Fusarium* resistance, were converted to KASPar assays then used to further validation on 96 susceptible and 96 resistant sugar beet samples. Fig. 4 shows allelic discrimination plots from competitive allele-specific PCR (KASPar) genotyping of the SNP_ Bv2_043450 and SNP_ Bv7_171470

polymorphisms. The genotyping call rates for both SNPs were 80% for resistant samples and 92% for the susceptible samples.



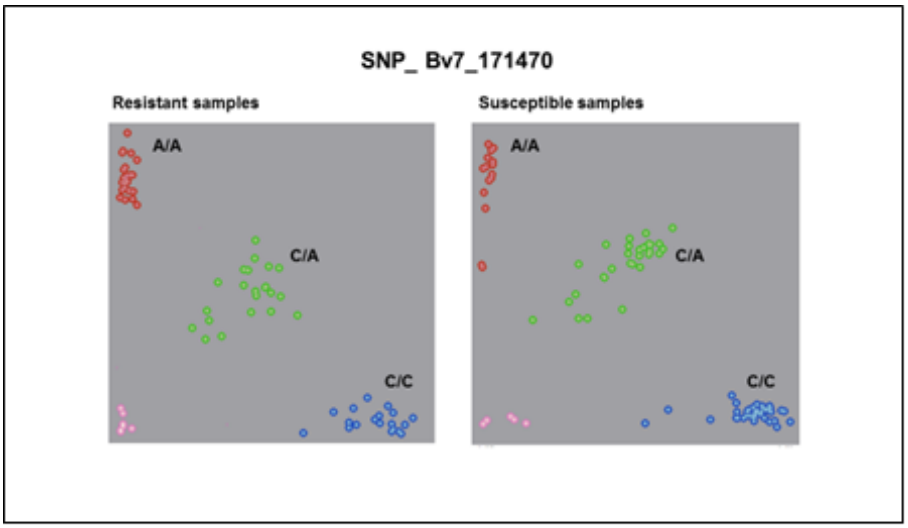


Figure 4: Allelic discrimination plots of SNP_ Bv2_043450 and SNP_ Bv7_171470 obtained by means of KASP analysis.

Table 2 showed the genotypic and allelic frequencies of SNP_Bv2_043450 and SNP_Bv7_171470 polymorphisms in 96 resistant and 96 susceptible samples.

The distribution of SNP_Bv2_043450 and SNP_Bv7_171470 genotypes were in Hardy-Weinberg equilibrium ($P<0.01$, data not shown) both in resistant and susceptible groups.

The frequency of the A allele both for SNP_Bv7_171470 and SNP_Bv2_043450 was significantly higher (P<0.05) in the resistant individuals than in susceptible individuals.

The distribution frequencies of the A and C alleles in the SNP_Bv2_043450 were 47% and 53% in the resistant group and 34% and 66% in the susceptible group, respectively. The AA, AC and CC genotypes frequencies were 25%, 44% and 31% in the resistant group and were 8%, 52% and 40% in the susceptible group, respectively.

Allelic distribution of SNP_Bv7_171470 for A and C alleles in the resistant group were 53% and 47% and in the susceptible group were 35% and 65%. The AA, CA and AA genotypes were 37%, 33% and 30% in the resistant group and 17%, 36% and 47% in the susceptible individuals, respectively.

Table 2: Comparison of genotypic and allelic distribution of SNP_Bv2_043450 and SNP_Bv7_171470 between resistant and susceptible groups.

SNPs	Genotypes [n(%)]			X^2	P value	Alleles [n(%)]		X^2	P value
	A/A (freq)	A/C (freq)	C/C (freq)			A (freq)	C (freq)		
SNP_ Bv2_043450									
Resistant	19 (24.7%)	34 (44.2%)	24 (31.2%)	8.69	0.012	72 (53.3%)	82 (46.7%)	5.48	0.019
Susceptible	7 (8%)	46 (52.3%)	35 (39.8%)			60 (34.1%)	116 (65.9%)		
SNP_ Bv7_171470									
Resistant	28 (36.8%)	25 (32.9%)	23 (30.3%)	8.57	0.013	81 (53.3%)	71 (46.7%)	10.4	0.012
Susceptible	15 (17.4%)	31 (36%)	40 (46.5%)			61 (35.5%)	111 (64.55%)		

For each SNP we calculated odds ratios (OR) and confidence intervals (CI) for the allelic association analysis by a logistic regression analysis testing multiple inheritance models (codominant, dominant, recessive, over-dominant, and log-additive). The best model was identified according to the smallest Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) values.

The genotype frequency of AA in the SNP_Bv2_043450 and in the SNP_Bv7_171470 were both significantly associated with *Fusarium* resistance (p =0.0029, OR =0.26,

95% CI = 0.10-0.67 and p= 0.0069, OR=0.36, 95% CI = 0.17-0.76). This suggests that the allele A of the SNP_Bv2_043450 (Table 3) and SNP_Bv7_171470 (Table 4) was associated with an increased *Fusarium* resistance. The model with the lowest AIC and BIC for both SNP_Bv2_043450 and SNP_Bv7_171470 was the recessive model.

Table 3: Different inheritance models analysis of SNP_Bv2_043450 between resistant and susceptible groups.

Model	Genotype	Resistant [n(%)]	Susceptible [n(%)]	OR (95%CI)	P-value	AIC	BIC
Codominant	C/C	24 (31.2%)	35 (39.8%)	1	0.012	225	234
	C/A	34 (44.2%)	46 (52.3%)	0.93 (0.47-1.84)			
	A/A	19 (24.7%)	7 (8%)	0.25 (0.09-0.69)			
Dominant	C/C	24 (31.2%)	35 (39.8%)	1	0.25	231	237
	C/A-A/A	53 (68.8%)	53 (60.2%)	0.69 (0.36-1.31)			
Recessive	C/C-C/A	58 (75.3%)	81 (92%)	1	0.003	223	229
	A/A	19 (24.7%)	7 (8%)	0.26 (0.10-0.67)			
Overdominant	C/C-A/A	43 (55.8%)	42 (47.7%)	1	0.3	231	237
	C/A	34 (44.2%)	46 (52.3%)	1.39 (0.75-2.56)			
Log-additive	---	---	---	0.58 (0.37-0.92)	0.018	226	233

OR= odds ratio, CI=confidence interval. Five inheritance models (codominant, dominant, recessive, over-dominant, and additive) were tested according to the Akaike information criterion (AIC) and Bayesian information criterion (BIC) using SNPStats tool. The inheritance model with the lowest values of AIC and BIC has the best fit.

Table 4: Different inheritance models analysis of SNP_Bv7_171470 between resistant and susceptible groups

Model	Genotype	Resistant [n(%)]	Susceptible [n(%)]	OR (95%CI)	P-value	AIC	BIC

Codominant	C/C	23 (30.3%)	40 (46.5%)	1	0.013	221	231
	A/C	25 (32.9%)	31 (36%)	0.71 (0.34-1.49)			
	A/A	28 (36.8%)	15 (17.4%)	0.31 (0.14-0.69)			
Dominant	C/C	23 (30.3%)	40 (46.5%)	1	0.034	223	230
	A/C-A/A	53 (69.7%)	46 (53.5%)	0.50 (0.26-0.95)			
Recessive	C/C-A/C	48 (63.2%)	71 (82.6%)	1	0.005	220	226
	A/A	28 (36.8%)	15 (17.4%)	0.36 (0.18-0.75)			
Overdominant	C/C-A/A	51 (67.1%)	55 (64%)	1	0.67	228	234
	A/C	25 (32.9%)	31 (36%)	1.15 (0.60-2.20)			
Log-additive	---	---	---	0.56 (0.38-0.84)	0.004	220	226

OR= odds ratio, CI=confidence interval. Five inheritance models (codominant, dominant, recessive, over-dominant, and additive) were tested according to the Akaike information criterion (AIC) and Bayesian information criterion (BIC) using SNPStats tool. The inheritance model with the lowest values of AIC and BIC has the best fit.

DISCUSSION

In this study, we used a candidate gene approach to identify SNP markers associated with *Fusarium* resistance in sugar beet. The results have demonstrated that the combination of candidate gene approach with two high-throughput and cost effective methods as HRM analysis and KASPar technology led to identify and validate SNP markers associated with *Fusarium* resistance.

Resistance genes to *Fusarium oxysporum* were previously identified in many crops (Simon et al. 1998, Sharma and Muehlbauer 2007) and several studies have been conducted to identify molecular markers linked to resistance (Mutlu et al. 2008, Oumouloud et al. 2008, Jain et al. 2015). For instance, one dominant and one recessive gene were identified for *Fusarium* resistance in pigeonpea (Saxena et al. 2012). Three dominant genes (Fom-1, Fom-2 and Fom-3) and a recessive gene (fom-4) were found in melon (Joobeur et al. 2004, Oumouloud et al. 2010). Recently, SNP markers linked to *Fusarium* resistant were found in *Physalis peruviana* (Enciso-Rodriguez et al. 2013) and in watermelon (Ren et al. 2015).

The two identified SNP mutations associated with *Fusarium* resistance were mapped in exon of the gene Bv2_043450_zhxk and in exon of the Bv7_171470_ojty gene, respectively. The gene Bv2_043450_zhxk encoded a CC-NBS-LRR disease resistance protein At4g27190 and Bv7_171470_ojty encoded a NBS-LRR putative disease resistance protein At3g14460. These two genes are both involve in defense response and ATP binding (Dohm et al. 2014). Plant NBS-LRR proteins mainly function in host resistance by specific interaction with pathogen effectors, which active defense response (Jones and Dangl, 2006). LRR domains play roles in interaction between gene and pathogen, while NBS domains is involved in blind ATP (Porter et al. 2009). CC domains are involved in protein-protein interaction and signaling (Martin et al. 2003, Marone et al. 2013). Therefore, it is possible that Bv2_043450_zhxk and Bv7_171470_ojty play an important role in *Fusarium oxysporum* resistance in sugar beet. Both SNP polymorphisms are silent mutations, which do not result in an amino acid exchange. While non synonymous SNPs can change protein structure and function, some synonymous SNPs might affect translational kinetics leading to an altered protein conformation and consequently change protein function (Kimchi-Safarty et al. 2007, Komar 2007). In addition, any non-functional SNP could be linked to functional variants.

This study is an initial achievement for future improvement of sugar beet against *F. oxysporum*. Host-plant resistance is the most economic and effective strategy for control of *Fusarium* diseases in sugar beet cultivars and for this reason the introgression of these resistant genes into susceptible materials is important to maintain the crop production.

In conclusion, our results suggest that the two SNP markers, SNP_Bv2_043450 and SNP_Bv7_171470 play a significant role in the *Fusarium* resistant. These SNPs can be used in breeding-assisted selection programs to improve *Fusarium* resistance in sugar beet.

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GENERAL CONCLUSIONS

The improvement of *Fusarium* resistance in sugar beet is important to overcome *Fusarium* infection in the field. To achieve this goal it is essential to screen the germplasm for *Fusarium* resistance and develop molecular markers linked to resistance for marker assisted selection.

The first part of this thesis provided the state of art of the sugar beet breeding achievements obtained so far, in order to provide an overview on traditional sugar beet breeding strategies and the development of molecular breeding methods. The progress in breeding techniques and technologies leads to rapidly evolving of new approaches and it is essential to investigate and determine the most appropriate method to apply in a sugar beet breeding program.

The phenotypic screening conducted in this work led to the identification of resistant lines to *Fusarium oxysporum* f. sp. *betae* that can be used in breeding programs to improve resistance. Moreover, it was observed for the first time that isolates of *F. oxysporum* f. sp. *betae,* from different genetic sub-groups, with various levels of pathogenicity, can cause not only vascular discoloration but also severe root rot. Therefore, Fusarium root rot symptoms in sugar beet appear to have a host component as well as a fungal strain component.

The candidate gene approach used in this study allowed to identify two SNP markers associated with the resistance in the exon of genes *Bv7_171470_ojty* and *Bv2_043450_zhxk*, respectively. These two genes are both involved in defense response and may play an important role in *Fusarium* resistance. The two SNPs can be used in molecular marker selection to improve *Fusarium* resistance in sugar beet. Moreover, the thesis has demonstrated that the combination of a candidate gene approach with two high-throughput and cost-effective methods, HRM analysis and KASPar technology, is an effective technique to identify and validate SNP markers linked to disease resistance. The feasibility of using this approach on other crops with available genome sequence represents the starting point to set up specific breeding programs to improve disease resistance.

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