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RESEARCH PAPER

Anti-proliferative and apoptosis inducing activity of *Calophyllum inophyllum* L. oil extracts on C6 glioma cell line

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Abstract

Glioblastoma multiforme (GBM) is one of the most lethal type of brain tumors. Current treatment methods of GBM including radiotherapy and chemotherapy are not sufficient to combat this disease and there is a great need to develop new treatment approaches. *Calophyllum inophyllum* L. is a polyphenol rich plant with wide biological activities. The aim of this study was to investigate cytotoxic, anti-proliferative and apoptotic activity of *C. inophyllum* oil (CIO) extracts on the C6 glioma cell line to determine its potential use in cancer treatment. Treatment of C6 glioma cells with CIO extracts for 24 and 48 h were resulted in a significant decrease in cell viability with IC_{50} values of 0.22% and 0.082%, respectively. Proliferating cell nuclear antigen labeling of CIO extract treated C6 glioma cells showed cell proliferation decreased to 55.6% and 30.3% for 24 and 48 h. Percentage of apoptotic cells after CIO extract treatment were found to reach 68.8% after 48 h. Analysis of mRNA expression levels of key genes involved in apoptosis pathway showed that CIO extract treatment induces both intrinsic and extrinsic apoptosis pathways on C6 glioma cells. Findings of this study showed that CIO extracts are promising in development of new treatment strategies for glioblastoma.

Introduction

Glioblastoma multiforme (GBM) is one of the most common and malignant primary brain tumor in adults. GBM grows rapidly and has a high rate of invasion and angiogenesis capacity but they often do not metastasize out of the brain. This tumor type carries the highest incident rate among brain and central nervous system tumors with the incident rate of 3.19/100.000 population (Thakkar et al., 2014). On the other hand, GBM has worst prognosis among other types of brain cancers (Hanif et al., 2017) . The median survival for GBM is 15 months and less than 5% of patients survive 5 years post-diagnosis (Delgado-López & Corrales-García, 2016). The standard treatment approach for GBM is very complex, it includes surgical resection of tumor followed by radiotherapy and chemotherapy (Delgado-López & Corrales-García, 2016). Despite optimal treatment, high resistance of tumor cells to chemotherapy and radiotherapy, GBM has very poor survival rate. Since conventional therapies not sufficient to combat this disease there is a great need to develop new treatment approaches.

Phytochemicals are natural plant derived compounds and known to be as a great source for development of many drugs. Many phytochemicals and their derivatives are known to have high antitumor potential for cancer treatment. In addition, it is known that phytochemical-based agents increase treatment efficiency and reduce most of the side effects of drugs

(Choudhari et al., 2020). Polyphenols are one of the most diverse phytochemical groups and exhibits wide range of biological activities including anti-cancer, anti-proliferative and anti-oxidant activities (Upadhyay & Dixit, 2015). *Calophyllum inophyllum* L. (Calophyllaceae) is a polyphenol rich evergreen pantropical tree found in Africa, Asia, and Pacific countries (Dweck & Meadows, 2002). Various parts of *C. inophyllum* plant such as barks, leaves, and fruits are used for different traditional medicinal purposes (Raharivelomanana et al., 2018). *C. inophyllum* contains wide range of phytochemicals such as triterpenoids, steroids, coumarins, and flavonoids (Susanto et al., 2019). Hence, *C. inophyllum* extracts are also known to have anti-oxidant, anti-microbial, anti-viral, anti-inflammatory and anti-proliferative activities (Dweck & Meadows, 2002). Nevertheless, studies carried out until today, mostly focused on the uses of *C. inophyllum* extracts in cosmetic industry and wound healing (Ansel et al., 2016; Raharivelomanana et al., 2018). Considering its wide biological activities *C. inophyllum* has a great potential to be used in pharmaceutical development, especially for use in cancer treatment. However, there are limited numbers of studies in the literature that have investigated the potential use of *C. inophyllum* extracts in cancer treatment (Hsieh et al., 2018; Jaikumar et al., 2016; Shanmugapriya et al., 2017).

Development of most phytochemical-based drugs begins with the *in vitro* investigation of anti-tumor potential of plant extract. In this study, we aimed to investigate cytotoxic, anti-proliferative and apoptotic activity of *C. inophyllum* oil extracts on the C6 glioma cell line in order to determine its potential use in cancer treatment.

Materials and Methods

Materials

Pure cold-press *C. inophyllum* oil (100%) (CIO) extract was purchased from EuropeVital Herbal and Aromatic Oil, Turkey. The C6 glioma cell line and L929 mouse fibroblast cell line were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Proliferating cell nuclear antibody (PCNA) was purchased from Thermo Scientific™, USA. ApopTag® Peroxidase In Situ Apoptosis Detection Kit was obtained from Millipore, USA. Direct-zol™ RNA MiniPrep kit (Zymo Research, Irvine, USA) was used for RNA isolation. SensiFAST™ cDNA Synthesis Kit Bioline Reagents Ltd., UK) was used for cDNA synthesis. SensiFAST™ SYBR Lo-Rox (Bioline Reagents Ltd, London, UK) kit was used for quantitative real-time polymerase chain reaction (qRT-PCR).

Cell Culture

The C6 glioma cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's Nutrient Mixture F-12 medium (1:1) containing 5% fetal bovine serum (FBS), streptomycin (100 µg/mL), penicillin (100u/ml) and l-glutamine (0.2 mM). L929 fibroblast cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's Nutrient Mixture F-12 medium (1:1) containing 10% fetal bovine serum (FBS), streptomycin (100 µg/mL), penicillin (100u/ml) and l-glutamine (0.2 mM). Cells were incubated at 37°C in a saturated humidity atmosphere with 5% CO_2.

MTT Cell Viability assay

The cytotoxic effect of CIO extract on C6 glioma and L929 fibroblast cell lines were determined using MTT (3–(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells were seeded on a 96 well plate at a density of $1x10^4$/well and incubated overnight. Next day, old media was removed and cells were treated with medium containing various concentrations of CIO and cells were cultured for 24 and 48 h. After 24 and 48 h incubation with CIO extract, supernatants were removed and MTT solution (50 µg/mL) was added on each well for 3 h incubation at 37°C. The amount of purple formazan was determined by dissolving it in 0.1 mL DMSO and measuring the optical density at 570 nm in a microplate reader. Untreated cells were used as control group. The cytotoxic effect of CIO extract against C6 glioma cells was expressed as IC_{50}. Cell viability (%) was calculated by the following equation:

$$Cell\ viability\ \% = \frac{OD\ value\ of\ treated\ cells}{OD\ value\ of\ control\ cells} x\ 100$$

Proliferating cell nuclear antibody (PCNA) cell proliferation assay

The effect of CIO extract treatment on C6 glioma cell proliferation was determined by immunocytochemical detection of the presence of PCNA antibody as previously described (Ersoz et al., 2019). C6 glioma cells were cultured on 24 well plates at a density of $1x10^4$ cells in wells containing coverslips. Cells were incubated until they reach confluency. Then, 24 and 48 h IC_{50} concentration of CIO extracts were applied on C6 glioma cells. After 24 and 48 h incubation, cells were washed with phosphate buffered saline (PBS). Cells were fixated by ice-cold methanol treatment for five minutes. Following fixation step, blocking solution was applied and cells were incubated with PCNA primary antibody (1:300) overnight at 4°C. After overnight incubation cells were washed again and biotinylated secondary antibodies, streptavidin, biotinylated horseradish peroxidase were applied on cells. AEC kit (Invitrogen, Camarillo, USA) was used to stain immunoreactive cells and hematoxylene was used as counterstain. Olympus BX-50 bright field microscope was used to visualize stained and unstained cells. 10 random fields were chosen, and stained/unstained cells were counted to determine the percentage of immunoreactive cells. Percentage immunoreactivity was calculated as follows:

$$immunoreactivity\ \% = \frac{\#\ of\ immunoreactive\ cells}{\#\ of\ total\ cells} \times 100$$

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

Immunocytochemical investigation of CIO extracts on C6 glioma cell apoptosis induction was determined by TUNEL assay according to manufacturer's protocol. C6 glioma cells were seeded on coverslips at a density of $1x10_4$ cell per well. Then, 24 and 48 h IC_{50} concentration of CIO extracts were applied on cells. Cells were fixated with ice-cold methanol followed by immediate addition of equilibration buffer. TUNEL reaction mixture (terminal deoxynucleotidyl transferase and reaction buffer with nucleotide mixture) was added on cells and incubated for 1 h at 37°C. Diaminobenzidine (DAB) as the substrate of peroxidase was used to stain the DNA fragments. 10 random fields were selected to count stained/unstained cells. Percentage of apoptotic cells was determined as follows (Ersoz et al., 2019):

$$apoptotic\ cells\ \% = \frac{\#\ of\ apoptotic\ cells}{\#\ of\ total\ cells} \times 100$$

Gene expression analysis by quantitative real-time polymerase chain reaction

C6 glioma cells were treated with IC_{50} concentration of CIO extracts for 48 h. Total RNA isolation from cells was performed using the Direct-zol™ RNA MiniPrep kit (Zymo Research, Irvine, USA), according to the manufacturer's instructions. After isolation, RNA integrity and concentrations were checked by using NanoDrop UV-Vis spectrophotometer. SensiFAST cDNA synthesis kit (Zymo Research, Irvine, USA) was used to synthesize cDNA from purified RNA and manufacturer's instructions were followed for synthesis. Briefly, 1 µg RNA, 4 µl 5x TransAmp Buffer and 1 µl reverse transcriptase enzyme were mixed and reaction mixture was completed to 20 µl with dH_2O. Then, this mixture was incubated 10 min at 25°C for primer annealing, 30 min at 42°C for reverse transcription and 5 min at 85°C for inactivation step in a thermal cycler. SensiFAST SYBR Lo-Rox kit (Bioline Reagents Ltd, London, UK) was used to detect gene amplification in qRT-PCR. Reactions were carried out using AriaMx Real-Time PCR System (Agilent Technologies, Santa Clara, USA). Reaction conditions were 2 min pre-denaturation at 95˚C, denaturation at 95˚C for 5 s, annealing at 63˚C for 10 s and elongation at 72˚C for 15 s for 40 cycles. Primer pairs of target genes and housekeeping β-actin gene were listed in Table 1. The relative gene expressions of all the genes tested were determined by using $2^{-\Delta\Delta Ct}$ method where β-actin gene was used as internal control (Livak & Schmittgen, 2001) (Table 1).

Statistical analysis

Graphpad Prism software version 6 (GraphPad Software, La Jolla, CA) was used for all statistical analysis. Unpaired t-tests were used for comparisons between groups, differences were considered to be significant at the level of $p<0.05$.

Results and Discussion

Determination of C6 glioma cell cfigureytotoxicity

Cytotoxic activity of CIO extracts on C6 glioma cells were determined by MTT assay. L929 fibroblast cells were used to determine CIO extract's cytotoxic activity on normal cells. C6 glioma and L929 fibroblast cells were treated with different concentrations of (0.02% to 1%) CIO extract for 24 and 48 h. CIO extracts inhibited viability of C6 glioma cells in a dose and time dependent manner. On the other hand, L929 cells treated with various concentrations of CIO extract did not change cell viability for both 24 and 48 h treatment (Figure 1a and 1b). As shown in Figure 1a, 24 h treatment of C6 glioma cells with CIO extracts did not show any significant cytotoxic activity in lower concentrations such as 0.02% and 0.05%. However, increasing concentrations of CIO started to inhibit proliferation of C6 glioma cells. The significant decrease in cell proliferation was observed at 0.125% concentration and IC_{50} was found 0.22% for 24 h treatment with CIO extract. L929 cell viability did not change even in the higher concentrations of CIO treatment (Figure 1a). The inhibitory effect of CIO extracts on C6 glioma cells were higher after 48 h treatment compared to 24 h (Figure 1b). Figure 1b represents inhibitory effect of CIO extracts on C6 glioma cells and L929 fibroblast cells after 48 h treatment. C6 glioma cell viability decreased to 55% after treatment with 0.05% CIO extracts. Cell cytotoxicity of C6 glioma cells were significantly increased at concentrations of 0.05% and above (Figure 1b). Treatment of C6 glioma cells with 1% CIO extract resulted in 11% cell viability.

Table 1. Specific primer sequences used for qRT-PCR

Gene	Forward Primer	Reverse Primer
β -actin	5'-CATGTACGTTGCTATCCAGGC-3'	5'-CTCCTTAATGTCACGCACGAT-3'
Cytochrome-c	5'-CTTTGGGCGGAAGACAGGTC-3'	5'-TTATTGGCGGCTGTGTAAGAG-3'
Caspase-3	5'-AGAGGGGATCGTTGTAGAAGTC-3'	5'-ACAGTCCAGTTCTGTACCACG-3'
Caspase-9	5'-CTCAGACCAGAGATTCGCAAAC-3'	5'-GCATTTCCCCTCAAACTCTCAA-3'
Caspase-8	5'-GTTGTGTGGGGTAATGACAATCT-3'	5'-TCAAAGGTCGTGGTCAAAGCC-3'
Bax	5'-CCCGAGAGGTCTTTTTCCGAG-3'	5'-CCAGCCCATGATGGTTCTGAT-3'
Bcl-2	5'-GGTGGGGTCATGTGTGTGG-3'	5'-CGGTTCAGGTACTCAGTCATCC-3'

IC_{50} concentration for 48 h treatment with CIO extract was calculated as 0.082%. Moreover, treatment with all concentrations of CIO extracts did not show any cytotoxic effect on L929 cells (Figure 1b).

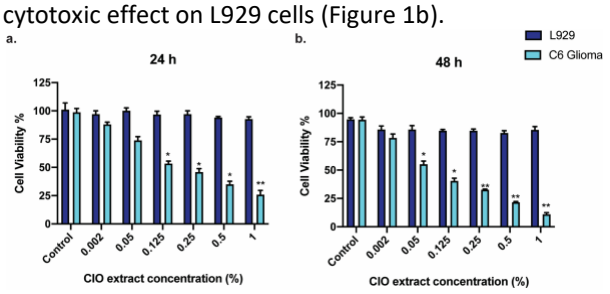

Figure 1. Percentage cell viability of L929 and C6 glioma cells after treatment with CIO extracts for **a)** 24h and **b)** 48 h. Data are expressed as mean ± standard deviation from three independent experiments. **p<0.05, ** p<0.001*.

Biological activity of C. *inophyllum* oil extracts were also previously tested in different studies. A study by Ansel et al. (2016) tested cytotoxic activity of C. *inophyllum* oil extracts cytotoxicty on human keratinocytes (HaCaT) and normal dermal fibroblasts (ECACC). In both cell lines, *C. inophyllum* oil extracts did not show any significant cytotoxic activity in a concentration range from 1% to 0.125%. Our results also revealed that CIO extracts were safe to be used in L929 fibroblast cells in the concentration range of 0.02% to 1%. The cytotoxic activity of CIO extracts was evaluated in different studies but most of these studies were focused on human skin cells (Ansel et al., 2016). However, literature on examining the impact of CIO extract on cancer cells were limited. A study investigating the in vitro anticancer of *C. inophyllum* ethanolic leaf extract on MCF-7 breast cancer cell lines found that IC_{50} of 120 µg/mL strongly inhibits MCF-7 cell growth. The same study was reported that the increase in cytotoxic activity of MCF-7 cells were also dependent on increasing concentrations of C. *inophyllum* ethanolic leaf extract (Léguillier et al., 2015).

CIO extracts inhibits C6 glioma cell proliferation

The effect of CIO extracts on C6 glioma cell proliferation was determined using PCNA immunocytochemical staining. C6 glioma cells were treated with IC_{50} concentrations of CIO extracts for 24 and 48 h. Then, immunostained cells were counted and PCNA positive cell rates were determined (Figure 2). CIO extract treatment significantly reduced C6 glioma cell proliferation both 24 and 48 h treatment. 24 h treatment with CIO extract resulted in 55.6% PCNA positive cells while 48 h treatment decreased PCNA positive cell numbers to 30.3% (Figure 2). Sustained proliferative signaling is one of the key hallmarks of cancer (Hanahan & Weinberg, 2011). Therefore, it is crucial to determine potential anti-cancer agents' effect on cell proliferation. It is known that phytochemicals are polyphenol rich natural compounds that derived from different plants (Upadhyay & Dixit, 2015). *C. inophyllum* is a polyphenol rich plant and contains many different biologically active groups such as flavonoids and coumarins. These chemical groups are individually a great source of potential anti-tumor agents (Itoigawa et al., 2001). On the other hand, studies suggest even minor compounds in oil extracts might have great importance in order to synergistically act and inhibit cancer cell proliferation (Bakkali et al., 2006). Our results revealed that CIO extracts inhibits cell proliferation in a time dependent manner on C6 glioma cells. This inhibition could either be a result of synergistic activity of CIO extract's functional groups or individual activity of flavonoids and coumarins.

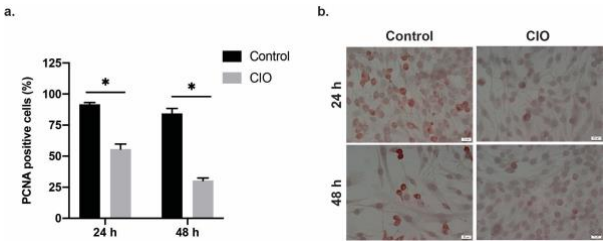

Figure 2. Effect of CIO extracts on proliferation of C6 glioma cells. **a)** PCNA positive cell rate of C6 glioma cells after 24 and 48 h treatment with IC_{50} concentration of CIO extracts. **b)** Bright-field microscope images of immunostained cells. **p<0.05.*

Determination of CIO extract induced apoptotic activity on C6 glioma cells by TUNEL assay

Immunocytochemical detection of CIO extracts apoptotic activity on C6 glioma cells showed by TUNEL assay. TUNEL method detects apoptotic cells that undergo extensive DNA degradation during the late stages of apoptosis (Kyrylkova et al., 2012). C6 glioma cells were treated with 24 and 48 h IC_{50} concentrations of CIO extracts. Results showed that CIO extracts induces apoptosis on C6 glioma cells (Figure 3).

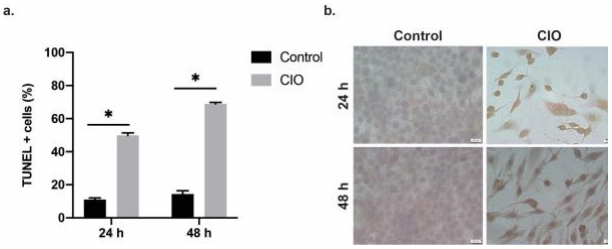

Figure 3. Detection of apoptotic C6 glioma cells treated with CIO extracts for 24 and 48 h. **a)** TUNEL positive labeled cell percentage **b)** Bright-field microscope images of immunostained cells. **p<0.05.*

Both 24 and 48 h incubation of C6 glioma cells with CIO extracts resulted in a significant increase in apoptotic cell number. TUNEL positive stained C6 glioma cell percentage was increased to 49.8% after 24 h treatment with CIO extracts. Moreover, apoptotic C6 glioma cell percentage reached 68.8% after 48 h treatment with CIO extracts. Untreated C6 glioma cells were used as controls. In contrast with CIO extract

treated cells, apoptotic activity did not change in control group for 24 and 48 h. Apoptosis is one of the most important events in cancer progression. Cancer cells are known for their resistance to apoptosis. Therefore, it is important to activate apoptosis in cancer cells in order to prevent tumor growth and cancer progression. Our results demonstrated on a cellular level that CIO extracts induces apoptosis on C6 glioma cells.

Determination of apoptosis related genes expression levels on CIO extract treated C6 glioma cells

TUNEL labeling assay results revealed that CIO extracts induces apoptosis on C6 glioma cells. In order to elucidate molecular base of CIO induced apoptosis induction, expression levels of key genes involved in intrinsic and extrinsic apoptosis pathway were investigated. For this aim, mRNA levels of cytochrome-c, caspase-3, caspase-9, caspase-8, bax and bcl-2 genes of CIO extract treated C6 glioma cells were determined by qRT-PCR. Since TUNEL labeling revealed 48 h incubation with CIO extracts increased apoptotic cell percentage to 68.8%, gene expression levels of apoptosis related genes were determined after cells treated with 48 h IC_{50} concentrations of CIO extracts. Differential gene expression levels were presented in Table 2. Results showed that CIO extract treatment induces both intrinsic and extrinsic apoptosis pathway on C6 glioma cells. Genes that activates intrinsic pathway of apoptosis were showed increased level of expression. Cytochrome-c is an important role to initiate intrinsic apoptosis pathway. Once it released from mitochondria to cytosol, it binds to apoptotic protease activating factor-1 and this event subsequently leads to formation of apoptosome complex (Arnoult et al., 2002). Our results showed that CIO extracts increased cytochrome-c expression 4.1 times than control.

Table 2. Relative mRNA expression levels of apoptosis related genes after CIO extract treatment on C6 glioma cells (*p<0.001*)

Gene	Relative Fold Change
β -actin	1
Cytochrome-c	4.1137
Caspase-3	4.3866
Caspase-9	6.2334
Caspase-8	2.3667
Bax	3.0157
Bcl-2	-0.8218

Release of cytochrome-c from mitochondria depends on Bcl-2 and Bax proteins activity. Bcl-2 protein binds pro-apoptotic Bax to prevent pore formation and release of cytochrome-c from mitochondria (Naseri et al., 2015). Expression level of Bax was 3.01 times higher than control cells. On the contrary, Bcl-2 relative mRNA expression was found to be -0.82 indicating the apoptosis activation and reverse relationship between Bcl-2 and Bax. Moreover, caspase-3 and caspase-9 expressions were also increased 4.3 and 6.2 times, respectively. Activation of caspase-9 also supports that CIO extracts induces intrinsic apoptosis pathway. Despite lack of literature on CIO extracts apoptosis inducing activity on cancer cells, one study established that treatment of DLD-1 human colon cancer cells with pigments from *C. inophyllum* seed oil resulted in apoptosis induction and G2/M cell cycle arrest (Hsieh et al., 2018). A study by Shanmugapriya et al. (2017) also revealed that treatment of MCF-7 breast cancer cells with *C. inophyllum* fruit extracts decreased Bcl-2 expression and increased Bax, cytochrome-c and p53 expressions. Taken together, our results were found to be consistent with the literature and it can be concluded that different extracts of *C. inophyllum* induces apoptosis on various cancer cell lines.

Conclusion

This study was aimed to investigate anti-proliferative and apoptosis inducing activity of CIO extracts on C6 glioma cells. Our results demonstrated that CIO extracts significantly reduced glioma cell viability. Additionally, immunocytochemistry studies showed that cell proliferation decreases, and apoptosis induction was increased after CIO treatment. Apoptosis induction was also confirmed by qRT-PCR analysis. Overall, our results suggest that CIO might have a potential use in cancer treatment. However, further *in vitro* and *in vivo* studies investigating the molecular mechanisms of the anti-cancer activity of CIO extracts are required.

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RESEARCH PAPER

Phenotypic and iPBS-retrotransposon marker diversity in okra (*Abelmoschus esculentus* (L.) Moench) germplasm

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Abstract

This study was undertaken to assess genetic and phenotypic diversity of Turkish okra (*Abelmoschus esculentus* (L.) Moench) germplasm of 26 landraces including three cultivars (Akköy-41, Kabaklı-11, and Marmara-1) with 34 phenotypic traits and 74 iPBS-retrotransposon primers. Leaf-blade size, fruit length, fruit diameter, fruit number per plant, petiole length, plant height, stem diameter, number of stem nodes, and plant growth type (degree of branching) were the most important morphological traits contributing to the variation. Comparison of genotypes with 14 iPBS-retrotransposon primers yielded 141 bands, 34 of which (24.1%) were polymorphic, with the primer 2271 producing the highest (6) bands per primer. Cluster analysis based on phenotypic and molecular markers produced two major groups. Phenotypic based unweighted pair group method with arithmetic mean (UPGMA) dendrogram had 12 sub-groups with the highest similarity (0.63) between GAN-19/GAN-21 and MGL-6/Akköy-41 genotypes. The markers, however, produced a dendrogram with eight subgroups, pairwise genetic similarities ranging from 0.43 to 1.00, where MGL-6 singled out with a similarity value of 0.57. Howbeit, the Mantel test between both dendrograms based on the similarity matrix was insignificant.

Introduction

The okra, a member of the Malvaceae family, is taxonomically known as *Abelmoschus esculentus* (the binominal name is *Abelmoschus esculentus* (L.) Moench) and by the synonym *Hibiscus esculentus* L. Although the geographical origin of okra is controversial, it is thought to be originated from a tropical region of Asia or Africa (Tindall, 1983). West Africa was considered okra native land because of the presence of related wild species in the Nile Valley and Ethiopia (De Candolle, 1886; Yildiz et al., 2015a). Genetic studies have shown that okra's origin may consist of many species from Southeast Asia, India, West Africa, or Ethiopia. It was grown by the ancient Egyptians in the 12th century B.C., and its cultivation spread to the Middle East and North Africa (Lamont, 1999). Okra is cultivated in different tropical, subtropical, and warm temperature regions of the world Karakoltsidis & Constantinides, 1975). Around 10 million tons of okra are produced worldwide, with most of its production in Asia and Africa. Today, India is the biggest okra producer with 6 million tons, followed by Nigeria, Sudan, and Mali, respectively. In Turkey, okra is annually grown on 5640 ha with a production of 29,111 tons (FAO, 2018). Because of the similarity of the okra germplasm in Turkey to the African germplasm, it is thought that Turkey's introduction to okra was due to the okras brought to Anatolia from the African continent (Duzyaman, 2009). Production in Turkey is widely with local types. Three commercial cultivars have been recently introduced in Turkey (TTSM, 2020).

In addition to having low essential fatty acid content, okra fruits contain important macro-microelements and vitamins (Al-Wandawi, 1983), and

also another study have shown that okra seed is rich in unsaturated fatty acids (linoleic acid etc.) and high protein quality (Savello, 1980). Okra is not very selective in terms of soil, grown in tropical, subtropical and temperate regions of the world. Although it is a self-pollinated species, cross pollination can also be seen due to bees (Lamont, 1999). And this cross-pollination provides genetic variation that allows local okra genotypes to better adapt to environmental conditions. The chromosome number was reported to be 2n=130 (Joshi & Hardas, 1956) but there are two kinds of okra genotypes diploid (2n = 2x = 60-70) and tetraploids (2n = 4x = 120-130) that were attributed to abnormalities in chromosome movement during the mitotic stage of cell division (Nwangburuka et al., 2011). It is an allopolyploid crop derived from the regular polyploid series with n=12 and has diploid chromosome number ranging between 72 and 144 (Datta & Naug, 1968).

Interspecific hybridization is possible mechanism in the genus of *Abelmoschus* (Reddy, 2015), furthermore, the highest variation is seen in *A. esculentus* species, otherwise called the Saudanien type (Kalloo & Bergh, 1993). It has been determined that there are very few or no distinguishing characteristics in the okra varieties in Turkey, Iran and India (Martin et al., 1981). Although okra in Turkey might differ from each other, it was determined that the original structure is preserved in okra germplasm resources (Duzyaman & Vural, 2002). In the study with genotypes of different origins, it was shown that the morphology of Turkish okra is similar to those originating from India and Africa (Duzyaman & Vural, 2000). Because of high phenotypic similarity among the germplasms of different origins, it becomes crucial to determine genetic variations using DNA markers. In this way, information on the genetic diversity required for the rational use of plant genetic resources can be obtained in the most reasonable way (Chakravarthi & Naravaneni, 2006).

It is crucial for the agricultural policies of the countries to determine the genetic diversity by using molecular methods to preserve and develop varieties and lines with high yield, quality and adaptability. In this context, molecular markers have proven to be powerful tool in revealing genetic relationship intra and interspecies. There are different studies assessed the genetic diversity using sequence-related amplified polymorphism (SRAP) (Gulsen et al., 2007), random amplification of polymorphic DNA (RAPD) (Aladele et al., 2008; Prakash et al., 2011), simple sequence repeat (SSR) (Kumar et al., 2017), inter simple sequence repeat (ISSR) (Yuan et al., 2014), inter-primer binding site (iPBS) (Yildiz et al., 2015a; Yaldiz et al., 2018; Barut et al., 2020), microsatellite (Ravishankar et al., 2018) and amplified fragment length polymorphism (AFLP) (Akash et al., 2013) molecular markers in okra.

Retrotransposons are hereditary materials that assume a significant function in the plant genome regarding evolution, which can change the position and number of duplicates in the plant genome (Finnegan, 1989; Ali et al., 2019). “At the ends of the retrotransposons are long terminal repeats (LTRs) that qualify as a potential tRNA primer binding site (PBS)” (Finnegan, 1989). The iPBS markers are based on amplification of the PBS of the reverse transcriptase (tRNA complement) in two transposes opposite and close to each other (Kalendar et al., 2010). This study aimed to determine the genetic diversity among different local and commercial okra genotypes using both phenotypic and DNA-based iPBS-retrotransposon markers.

Materials and Methods

Genotypes and cultivars

Twenty-six okra genotypes of A. *esculentus* (L.) collected from different locations in Turkey were evaluated in this study (Figure 1; Table 1). Of these genotypes 20 okra accessions (BLK-1, MGL-2, MGL-3, MGL-4, MGL-5, MGL-6, MGL-7, MGL-8, MGL-9, MGL-10, AYD-11, AYD-12, AYD-13, MGL-14, UIS-15, UIS-16, USK-17, AYD-18, GAN-19, GAN-21) were local genotypes collected by farmers from different locations in Turkey. Two (YLV-22 and YLV-23) were advanced breeding lines developed by Ataturk Central Horticultural Research Institute (ACHRS), Yalova. The list also included 3 commercially registered cultivars (Akköy-41, Kabaklı-11 and Marmara-1) obtained from ACHRS and one standard commercial type (STD-20).

Figure 1. Okra genotypes and cultivars investigated in the experiment (numbers refer to row number in Table 1).

Table 1. Okra germplasm, accession number, place of origin, source and their local names

Row No	Accession No	Geographical Source	Source	Local Name
1	BLK-1	Gömeç/Balikesir	PC[a]	Local
2	MGL-2	Fethiye/Muğla	PC	Local
3	MGL-3	Fethiye/Muğla	PC	Local white
4	MGL-4	Fethiye/Muğla	PC	Local red
5	MGL-5	Fethiye/Muğla	PC	Local yellow
6	MGL-6	Seydikemer/Muğla	PC	Local mixed
7	MGL-7	Seydikemer/Muğla	PC	Local
8	MGL-8	Seydikemer/Muğla	PC	Local
9	MGL-9	Seydikemer/Muğla	PC	Local red
10	MGL-10	Seydikemer/Muğla	PC	Local
11	AYD-11	Aydın	PC	Local
12	AYD-12	Aydın	PC	Local
13	AYD-13	Sazlı/Aydın	PC	Local
14	MGL-14	Köyceğiz /Muğla	PC	Endeze
15	UIS-15	Ortaca/Muğla	PC	Local
16	UIS-16	Ortaca/Muğla	PC	Local
17	USK-17	Karahallı/Uşak	PC	Sultani
18	AYD-18	Köşk/ Aydın	PC	Tasbatan
19	GAN-19	Gaziantep	PC	Local
20	STD-20	Konya	SUNAGRI	Sultani
21	GAN-21	Gaziantep	PC	Local
22	YLV-22	Yalova	ACHRS[c]	ABL[b]
23	YLV-23	Yalova	ACHRS	ABL
24	Akköy-41	Yalova	ACHRS	CC[d]
25	Kabaklı-11	Yalova	ACHRS	CC
26	Marmara-1	Yalova	ACHRS	CC

[a]PC Personal Collection, [b]ABL Advanced breeding Line, [c]ACHRS Ataturk Central Horticultural Research Institute, Yalova, Turkey, [d]Commercial Cultivar

Production of plant material

Seeds collected from each genotype were sown at Akdeniz University Faculty of Agriculture Experiment Farm. The soil was off clay loam texture with a slightly alkali pH of 7.62, a high lime content of 17.7%, good organic matter content of 2.1%, total N content of 0.09%, low P content (0.0013%), high K content (0.19%), high Ca content (0.4%), optimum Mg content of (0.09%) and Mn, Zn, Cu and Fe contents respectively 2.67, 0.47, 0.25 and 1.2 mg/kg respectively. Irrigation was applied as required with dripping system, standard fertilizer was applied, and hand weeding was performed during the plant growth period.

Phenotypic/Agro-morphological traits measured

The phenotypic data of okra (*A. esculentus* (L.) Moench) were collected according to the criteria in the guidelines for conducting the distinctness, uniformity and stability described by International Union for the Protection of New Varieties of Plants (UPOV, 1999). A total of 34 traits were measured, including 7 agro-morphological traits (seed yield per plot (SYP), seed weight per pod (SWPP), seed yield per plant (SYPP), thermal time requirements for flowering (TTRF), thermal time requirements for fruit formation (TTRFF), plant growth type (PGT) and suitability to mechanical harvest (STMH)). TTRF and commercial harvest time were calculated in degree days by average of the daily greatest and least temperatures recorded and compared to a base of temperature, 10°C (Dhankar & Singh, 2013).

DNA extraction

Leaf samples from each genotype were collected from individual healthy green plants for molecular marker analysis. Genomic DNA was extracted from 40–50 mg young fresh leaf tissue of individual genotypes, using 2% CTAB method (Doyle & Doyle, 1987) and all DNA concentration of each genotype was measured using 1% agarose gel.

PCR amplifications with iPBS-retrotransposon markers

PCR amplifications were performed under reaction conditions containing 50 ng DNA template, 0.2 mM of each dNTPs (Thermo Fisher Scientific, USA) 0.2 µM of each iPBS primers, 1X Taq Polimerase Buffer (from 10X stock ammonium buffer with 15 mM $MgCl_2$), 1.25 U/µl Taq DNA polymerase (Ampliqon, Denmark) in the final volume using the following PCR conditions; 5 minute (min) denaturation at 95°C and 35 cycles of 30 second (s) denaturation at 95°C, 45 s annealing at 40-60°C, and 1 min extension at 72°C, followed by a final extension at 72°C for 10 min. The PCR products separated on 1X TBE Buffer (90 mM Tris, pH 8.3, 90 mM boric acid, 0.1 mM EDTA) with 2% agarose gels (Sigma-Aldrich Chemie GmbH, Germany) containing 0.5 µg/ml ethidium bromide (EtBr) and imaged under U.V. imaging system (DNR, Minilumi, Neve Yamin, Israel).

Data analysis

Polymorphic bands obtained by iPBS-retrotransposon primers used in the study were scored as present (1) or absent (0) due to the dominant nature of marker system. A dendrogram was created in "Numerical Taxonomy Multivariate Analysis System (NTSYS-pc)" version 2.1 software package (Rohlf, 1992) using UPGMA method (Michener & Sokal, 1957) to examine genetic relationships between genotypes. To create a dendrogram, DICE coefficient was based on the similarity matrix (Dice, 1945). Principal component analysis (PCA) was performed based on Jaccard similarity coefficient and two and three-dimensional graphs were created using the DCENTER and EIGEN procedures in NTSYS. Analysis was also made on phenotypic/agro-morphological data using SPSS statistical package (IBM, 2015) according to augmented statistical design. Similarity index was calculated and distance cluster dendrogram was created with S.M. coefficient and UPGMA method using NTSYS-pc ver. 2.1 software package. The eigen value of the phenotypic/agro-morphological data was calculated based on the similarity coefficient and of the variation explained. Comparison of marker and agro-morphological data based on similarity matrices with Mantel test (Mantel, 1967) was performed.

Results

Variation in phenotypic/agro-morphological traits

Considerable variation was recorded for all the qualitative and quantitative plant traits investigated

(Table S1). Higher variation was observed in quantitative traits compared with qualitative traits (Table S1). Variance was the highest in SYP (26273.7 g^2) followed by TTRFF (2572.6-degree day^2), and TTRF (2421.4-degree day^2), SYPP (193.2 g^2), petiole length (PL) (47.8 cm^2), diameter of young fruit (FD) (29.2 cm^2) and length of mature fruit (LMF) (25.3 cm^2). There were 3-fold difference in plant height (PH), ranging from 40.0 cm (GAN-19, MGL-7) to 120 cm (MGL-9) whereas stem diameter (SD) ranged from 4.58 cm (AYD-13) to 9.43 cm (MGL-9) (Table S1). FD ranged from 10.57 mm (BKL-1) to 26.69 mm (AYD-18) while diameter of mature fruit (DMF) ranged from 16.86 mm (Akköy-41) to 32.18 mm (UIS-16). Time of flowering (TF) was the earliest in MGL-9 and BLK-1 (55 and 58 days) and the latest in USK-17 and GAN-21 (68 vs 72 days, respectively). The earliest harvest was obtained from MGL-9 (61 days), YLV-23 (62 days) and BLK-1 (63 days). The highest number of fruits was obtained from Marmara-1 (16) and MGL-8 (14). The fruit number per plant (FNP) ranged from 3 (AYD-11, AYD-12, MGL-6, MGL-7 and GAN-21) to 16 (Marmara-1) with a mean of 6.23 (Table S1).

PCA with 34 traits produced 9 components with eigen values explaining 85.8% of total variation where the first 2 explaining 23.4% and 17.1% of variance, respectively (data not presented). The first three components explained 52.9% of cumulative variance. Leaf blade size (LBS) (0.793), SYP (0.756), flower size (FS) (0.754), (FNP) (0.695), PH (0.657), LMF (0,653), SD (0,639) and fruit thickness of carpel (TFC) (0.603) had the highest contributions in the first component whereas SWPP (0.658), FD (0.608), stem number of nodes (NSN) (0.603) and seed yield per plant (SYPP) (0.602) had the highest values in the second component as opposed to PGT (plant growth type) (0.721) having the highest value in the third component (data not presented).

The biplot for PCA of the first two components discriminated 4 groups of traits that distinctly comprised plant for quantitative (PH, SD etc.), color (fruit color (FC), stem color (SC) etc.), shape (fruit shape of apex (FSI), fruit constriction of basal part (CFBP) etc.) and phenological traits (TF, TFH etc.) (Figure 2). Shape and color traits were closely related while phenological and quantitative traits were more clearly separated (Figure 2). Shape and color traits are grouped on the negative part of X and Y axis while quantitative traits were positioned on the positive scale of X and Y axis. Time related traits were positioned on Y axis (Figure 2). NSN, STMH, DMF, TFC, FNP, PL, FS, LBS, plant growth type (PDB), petiole diameter (PD) and number of fruit locules (NFL) appeared to be the most important agro-morphological traits positively relating to yield (SWPP, SYPP and SYP).

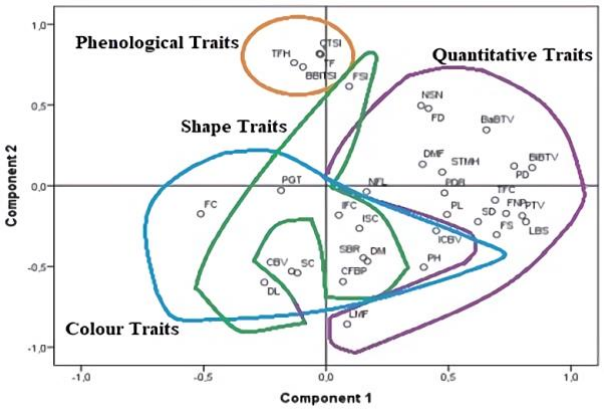

Figure 2. Biplot and PCA on phenotypic/agro-morphological traits investigated of okra genotypes and cultivar.

Cluster analysis based on qualitative and quantitative phenotypic traits

In line with cluster analysis of 27 phenotypic data described by UPOV, the dendrogram was created according to the UPGMA method and as a result, the existence of two major groups and different sub-groups under these major groups were determined. The highest similarity (0.63) was seen between GAN-19/GAN-21 genotypes collected from Gaziantep region and MGL-6/Akköy-41 genotypes. MGL-2 and MGL-9 genotypes formed a separate group in the dendrogram (Figure 3). The first two eigen values explained 21.05% of the total variation. In addition, 2D/3D plots of agro-morphological data were obtained by PCA analysis (Figure 4; Figure 5).

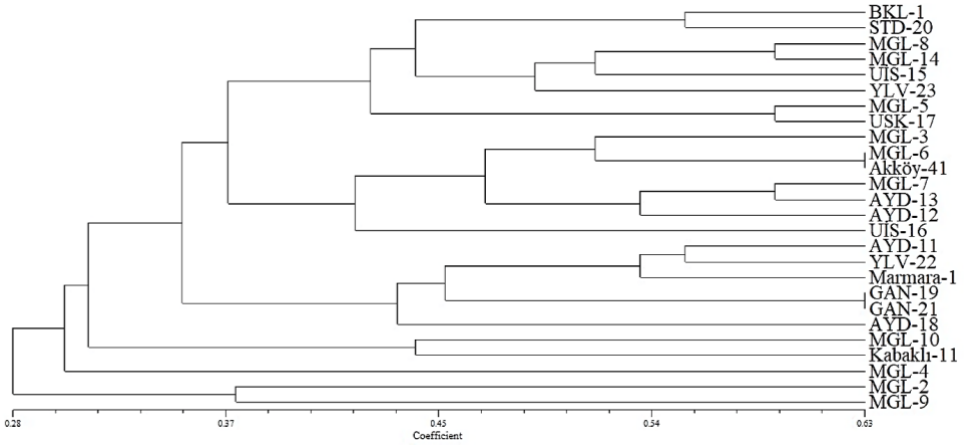

Figure 3. UPGMA similarity dendogram of local okra ecotypes based on qualitative and quantitative phenotypic traits.

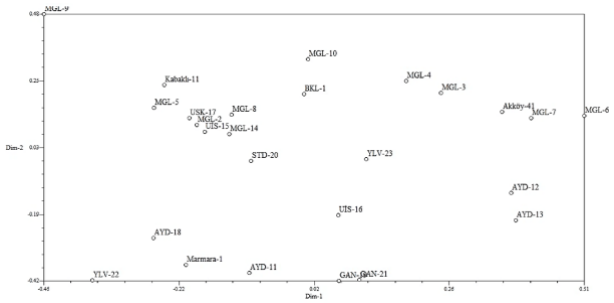

Figure 4. Two-dimensional plot of PCA of 26 okra genotypes based on phenotypic traits. Dim-1 and Dim-2 explain 10.76% and 10.29% of variation, respectively.

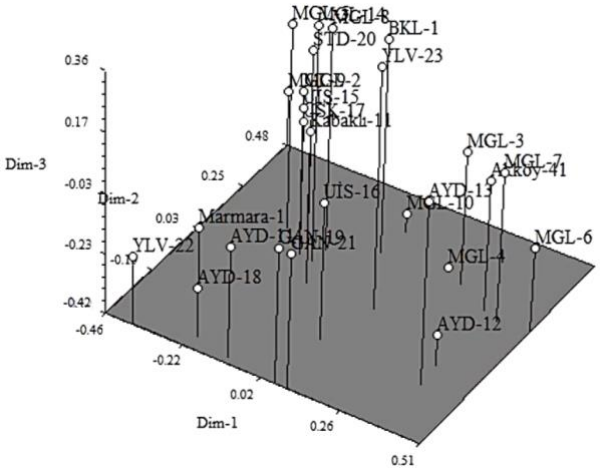

Figure 5. Three-dimensional plot of PCA of 26 okra genotypes based on phenotypic traits. Dim-1, Dim-2, and Dim-3 explain 10.76% and 10.29% and 8.25% of variation, respectively.

Comparison of genotypes with iPBS-retrotransposon primers

Initially, 74 iPBS-retrotransposon primers were used to screen the bulk DNAs of okra genotypes for amplification. Then 14 iPBS-retrotransposon primers were determined yielding polymorphic and highly reproducible bands. In this study, a total of 26 okra genotypes collected from different locations in Turkey were analysed with 14 different polymorphic İPBS-retrotransposon primers to determine the genetic relationship between genotypes.

At the same time, characterization study was carried out according to various criteria, and the data obtained were compared with iPBS marker and the correlation between the two dendograms was determined by the Mantel test. The 14 primers (2271, 2379, 2095, 2393, 2238, 2388, 2243, 2249, 2087, 2270, 2390, 2272, 2382, 2384) yielded 141 bands, 34 of which (24.1%) were polymorphic. One to six polymorphic band was obtained per primer. The primer 2271 yielded the highest (6) while the primers 2390, 2272, 2382, 2384 produced the lowest number of polymorphic bands (1) per primer. Polymorphic band sizes ranged from 230 to 2200 bp in size while the average number of bands and polymorphic markers per primer was 10.07 and 2.42, respectively (Table 2).

Cluster Analysis Based on iPBS-retrotransposon Markers

As a result of the dendrogram created, the existence of 2 different major groups was determined. Pairwise genetic similarities ranged from 0.43 to 1.00 and maximum similarity value of 1.00 were observed between 4 genotypes (MGL-3, MGL-9, AYD-11, AYD-13). iPBS markers failed to separate the four genotypes from each other. MGL-7 genotypes created a different group, separating from the rest in dendrogram with 0.57 genetic similarity. The remaining 25 genotypes were distinguished from one another with similarity values ranging from 0.57 to 1.00. Among the 25 genotypes, the MGL-6 genotype differed with a similarity value of 0.57 (Figure 6). The first two eigen values (2.63 and 1.86, respectively) obtained with PCA explained 36.1% of the total variation. The 2D and 3D graphs showed a similar result with dendrogram and most of the genotypes clustered together, especially MGL-3, MGL-9, AYD-11, AYD-13 genotypes with similarity values of 1.00 were clustered in a close area with STD-20 cultivars in this

Table 2. Genetic analysis of 14 polymorphic iPBS-retrotransposons markers with 26 Okra genotypes

Primers	Sequence	Tm (°C)	Total Band Numbers	Polymorphic Band Numbers	Product Sizes for Polymorphic Bands (bp)
2271	GGCTCGGATGCCA	54.3	11	6	500-2000
2379	TCCAGAGATCCA	41.5	9	4	400-1500
2095	GCTCGGATACCA	44.8	11	3	280-600
2393	TACGGTACGCCA	47.1	10	3	400-650
2238	ACCTAGCTCATGATGCCA	55.5	10	3	260-1250
2388	TTGGAAGACCCA	43.4	10	3	300-1250
2243	AGTCAGGCTCTGTTACCA	54.9	11	2	1900-2200
2249	AACCGACCTCTGATACCA	54.7	11	2	500-2000
2087	GCAATGGAACCA	43.5	10	2	230-250
2270	ACCTGGCGTGCCA	56.9	10	2	600-800
2390	GCAACAACCCCA	47.6	11	1	600
2272	GGCTCAGATGCCA	50.5	7	1	260
2382	TGTTGGCTTCCA	44.9	11	1	1000
2384	GTAATGGGTCCA	40.9	9	1	400
Total			141	34	
Average			10.07	2.42	

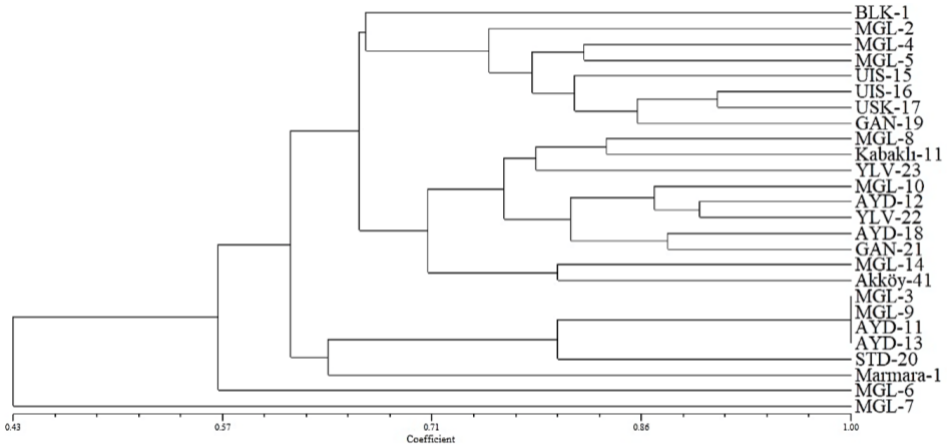

Figure 6. Dendrogram of 26 okra genotypes constructed by UPGMA based on İPBS-retrotransposon markers.

graph and formed a separate group. MGL-7 was located on the graph in a region separate from the group clusters (Figure 7; Figure 8).

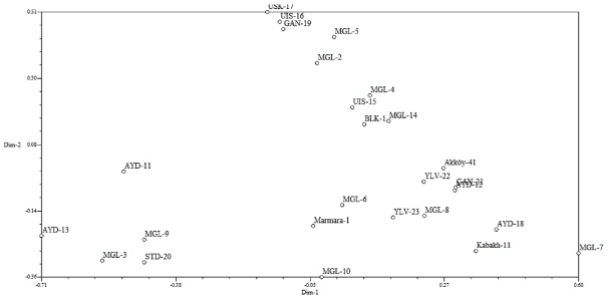

Figure 7. Two-dimensional plot of PCA of 26 okra genotypes based on iPBS-retrotransposon markers. Dim-1 and Dim-2 explain 21.12% and 14.98% of variation, respectively.

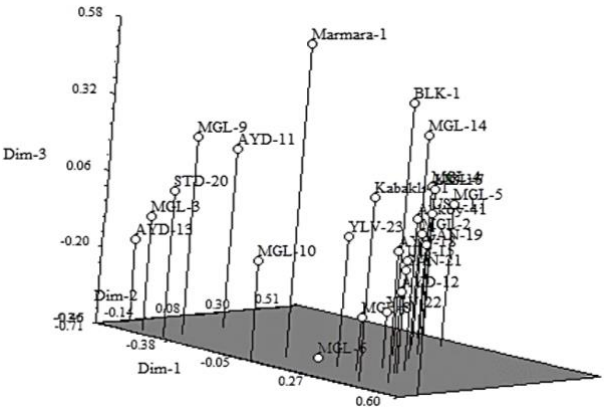

Figure 8. Three-dimensional plot of the principal component analysis (PCA) of 26 okra genotypes based on iPBS-retrotransposon markers. Dim-1, Dim-2, and Dim-3 explain 21.12% 14.98% and 9.86% of variation, respectively.

Discussion

The okra germplasm including 3 commercial cultivars showed considerable variation in the qualitative and quantitative plant growth descriptive traits investigated. LBS, FL, FD, PL, FNP, PH, SD, NSN and PDB were the most important quantitative characteristics contributing to variation within the germplasm. Yildiz et al. (2015b) working with 30 okra genotype and 35 morphological traits reported that fruit, leaf, stem color, leaf shape, flower size, fruit-surface angularity, petal color were important characteristics to distinguish the okra accessions.

Duzyaman (2005) working with 11 Turkish genotypes and 25 morphological, horticultural and physiological characteristics found that number of locules, mature pod length and internode length, pod yield/plant, pod number/plant, pedicel length and number of branches were main traits contributing the variability. Days to first flowering, nodes per plant, length of internode, fruit weight and fruits per plant were the most important traits contributing to heterosis in okra breeding (Bhatt et al., 2016). The higher number of accessions and descriptors were suggested in order to characterise better the representative regional morphotypes (Martin et al., 1981). Earliness and fruit yield as well fruit size and fruit color are important traits for consumers and producers. The germplasm we investigated had considerable variability for the traits that may serve as genetic material for a breeding program. The germplasm included landraces with thinner fruits (STD-20), short but thicker fruits, late maturing (GAN-21), determinate growth type (AYD-13), shortest plant height (MGL-7), thinner fruit diameter (MGL-6), shortest plants and fruits (GAN-19), highest seed yield (MGL-2), late, highest seed yield (USK-17), low fruit diameter (UİS-15), wider fruit diameter, easy harvest brittle fruit stems (UİS-16), dark green fruits (AYD-18), early, taller plants, long fruits (MGL-9), wide fruit diameter (YLV-22), taller plants and fruits (BKL-1), high seed yield (MGL-14) and earliness, higher fruit number (MGL-8). Although it was not a planned descriptive trait, brittleness of fruit stems discerned during harvest of UIS-16 could be an important quality for developing easily harvestable cultivars while dark green fruits of AYD-18 and very early flowering MGL-9 may contribute to genetic variation. Small okra pods of 2-4 days (<4 cm) are preferred largely in Turkey (Duzyaman & Vural, 2003b) whereas larger pods (>7-8

cm) are marketed in North America and India (Duzyaman & Vural, 2003a). Slower fruit fibre development in some cultivars (Kyriakopoulou et al., 2014) may be suitable for marketing long pods. Further work is required for the investigation of late fibre development properties of genotypes in Turkish germplasm in order to extend marketability of okra. This quality may compensate disadvantages of early harvest failure and exploit fruit yield potential of okra genotypes. Cultivars with earliness, easily harvestable traits, higher yield and superior market value may boost commercial potential of okra. Assessing genetic diversity of germplasm is crucial before establishing successful breeding programs.

Although the difference between okras in Turkey is usually determined by morphological properties, we have shown that iPBS markers can contribute to differentiate okra genotypes. Hence, both molecular and qualitative-quantitative agro-morphological traits were used to genotype and phenotype the 26 okra genotypes collected from different locations in Turkey. iPBS-retrotransposon marker systems, previously used to define the relationship between genotypes in different plant species (Barut et al., 2020; Yaldiz et al., 2018). Polymorphism was obtained for 26 okra genotypes using 14 of the 74 iPBS-retrotransposon primers used in the study. The polymorphism rate was 24.11%. At the same time, significant phenotypic variations were identified in terms of different characters handled within the local okra genotypes from different locations. Dendrograms created using molecular and agro-morphological data showed that two major groups exist among the genotypes. As a result of marker-data analysis, MGL-3, MGL-9, AYD-11, AYD-13 and also STD-20 genotypes are similar in terms of both dendogram and 2D/3D graph. The first two eigen values explained 36.1% of the total variation. Based on agro-morphological clustering, genotypes collected from similar location were determined to be located close to each other in the dendrogram. The highest similarity (0.63) was obtained with the GAN-19 and GAN-21 genotypes collected from the same location. Akköy-41 and Marmara-1 varieties were in a separate group than Kabaklı-11. However, in the dendrogram and 2D/3D graphs created by iPBS-retrotransposon molecular marker system, Kabaklı-11 and Akköy-41, developed from Sultani, were in the main group with the Sultani type standard variety (STD-20), while Marmara-1, a plump variety, was in the other group. The first two eigen values obtained as a result of analysis of agro-morphological data explained 21.05% of the total variation. Correlation between marker-based and agro-morphological-based dendrogram created according to similarity matrix was determined with Mantel test, was, however, insignificant (r = -0.11).

Marker-assisted molecular studies on okra are rare in the world. Previously, there were only two molecular marker studies with okra genotypes found in Turkey where SRAP, iPBS and SSR molecular markers were used (Gulsen et al., 2007; Yildiz et al., 2015a). At the end of a study using 66 okra genotypes and 13 polymorphic İPBS-retrotransposon primers, 88 bands and 40.2% total polymorphism rates were obtained as a result (Yildiz et al., 2015a). Higher total polymorphism rates of Yildiz et al. (2015a) probably originated from higher number of genotypes tested.

Gulsen et al. (2007) using 31 Turkish and 2 randomly selected U.S. genotypes and applying 39 SRAP primer combinations obtained approximately 50% of the 97 bands obtained which were polymorphic for 23 genotypes. It was determined that 17 of these 23 genotypes were separated from each other with an average of 0.93 similarities. However, although the UPGMA dendrogram based on 33 phenotypic markers distinguished all genotypes, the geographic relationship between okra genotypes was not detected.

In another study with 44 okra genotypes collected from different locations in India, a total of 104 bands were obtained with 14 RAPD primers randomly amplifying the genome, and 74.03% polymorphism was obtained. These 14 primers were found to make a clear distinction between genotypes (Prakash et al., 2011). The reason for the higher rate of polymorphism obtained in their study was the fact that main centre of origin of okra is Indian subcontinent with considerable variation. Another reason for the higher polymorphism rates obtained is thought to be the use of the RAPD molecular marker system, which probably provides random amplification to produce a higher number of bands. Work on the assessment of okra germplasm is not sufficient and hence further studies are required with higher number of landraces, accessions, lines and cultivars representing major okra growing areas and dominant types in Turkey. Many of the previous work cited varied in the traits investigated. Further studies should apply wider number of marker systems and commercial cultivars as well as morphological descriptors universally used by breeders of okra plant. It was reported that genetic base of Turkish okra germplasm is narrow requiring inclusion of external diversity to national collections to increase variability (Yildiz et al., 2015b). Because of this narrow genetic base, polymorphism detected by limited number of molecular marker systems was low and hence further work is required with the inclusion of the external germplasm having the qualitative and quantitative traits scarce in Turkish national gene pool and testing new marker systems and phenotypic markers.

Conclusion

LBS, LMF, FD/DMF, PL, FNP, PH, SD, NSN, and PDB were the most important characteristics contributing to the variation in the okra germplasm. The 14 iPBS primers yielded 141 markers, 34 of which (24.1%) were polymorphic. Cluster analysis based on phenotypic and iPBS-retrotransposon data produced two major groups with 12 and 8 subgroups respectively without any

significant correlation between the two in Mantel test. Selection of parents in okra breeding program by phenotypic descriptors in conjunction with iPBS-retrotransposon marker system may aid better selection for conservation and breeding studies.

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Author Contribution

FK: Conceptualization, Data Curation, Formal Analysis, Methodology, Project Administration, Resources, Supervision, Validation, Visualization, Writing – review and editing; SNY: Formal Analysis; CB: Formal Analysis, Investigation, Visualization, Writing – Original Draft Preparation; NY: Formal Analysis, Data Curation; and NM: Conceptualization, Methodology, Project Administration, Supervision, Validation, Writing – review and editing.

Additional Information

Supplementary data accompanies this paper at http://biotechstudies.org/uploads/BIO-132_Supp1.pdf

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BIOTECH STUDIES

RESEARCH PAPER

Isolation of cellulolytic fungi and their application for production of organic fertilizer from water hyacinth (*Eichhornia crassipes*)

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Abstract

The purpose of this study is to isolate the cellulolytic filamentous fungi involved in the decomposition of water hyacinth to produce organic fertilizer. In this study, 13 different fungi were isolated and screened. Among them, isolate N.S8 showed the highest cellulase activity with the diameter of the clear zone of 35 mm. The isolate N.S8 was identified by sequencing the D1/D2 region of 28S rRNA coding gene. BLASTN analysis of sequenced 28S rRNA segment revealed that the isolate N.S8 is *Aspergillus oryzae* with identity value and E-value of 100% and 0.0, respectively. Additionally, culturing the isolate N.S8 in rice bran medium (pH 6.5) for 144 hours is the optimal method of improving cellulase activity. Moreover, the use of this isolate for composting water hyacinth created an organic fertilizer with nitrogen (N), phosphorus (P_2O_5), and potassium (K_2O) contents of 3.35%, 0.43% and 0.74%, respectively after 45 days. Because of the high contents of nutrients, this organic fertilizer could solve the problems of fertilizer for crops with an efficiency being equivalent to that of chemical fertilizers which are known as one of the causes of soil degradation, environmental pollution and the negative effect on the quality of agricultural products.

Introduction

Fungi play an important role in the ecosystem by involving in nutrient cycling and energy flows (Buee et al., 2009). Fungi such as saprophytes convert organic matters in the soil into inorganic nutrients which plants could absorb (Morris et al., 2005). Many fungi which could successfully grow on various types of substrates and help to decompose organic matters to beneficial inorganic nutrients have been investigated (Fuchs et al., 2010).

Cellulolytic fungi are considered as the key factors to create high quality compost (Hubbe et al., 2010). Previous research indicated that the application of cellulolytic fungi increased the ability of holding water in fungal inoculated samples and balanced the C:N ratios of cellulose waste (Hart et al., 2002; Sivaramanan et al., 2014). Additionally, the addition of microorganisms such as fungi, bacteria and other microbes was demonstrated to shorten the composting duration (Mishra et al., 2013).

Water hyacinth (*Eichhornia crassipes*) is a free-floating freshwater plant of the family Pontederiaceae that has a cosmopolitan distribution but are mostly found in sub-tropical and tropical countries (Agunbiade et al., 2009; Jafari et al., 2010). Water hyacinth has been proven to be a highly problematic invasive species due to its detrimental impacts of preventing sunlight and lowering disolved oxygen concentration. Controlling this plant is difficult because of its fast growth and large biomass (Akter et al., 2009). Therefore, there is a great attention of treating water hyacinth biomass.

Water hyacinth can be processed into compost (Zimmels et al., 2006). Previous studies demonstrated that compost produced from water hyacinth is good for the yields and growths of many plants (Osoro et al., 2014). Additionally, the supplement of fungi such as *Trichoderma* for composting water hyacinth was proved to be useful for improving nutrition quality (Ghosh et al., 2010).

The purpose of the present research is to introduce some results about the selection of cellulolytic fungi and its application for the production of organic fertilizer from water hyacinth to create a high-quality organic fertilizer source that could increase the porosity, soil fertility and alleviate environmental pollution.

Materials and Methods

Sample collection

Soil samples (30 samples) were collected from several areas including gardens and fields in Thua Thien Hue, Vietnam. Sterile spatula and plastic bags were used for sample collection. Before being used for fungi isolation process, the samples were stored at 4 ^{o}C for 12 hours (Singh et al., 2013).

Water hyacinth (*Eichhornia crassipes*) was obtained from river and lakes in Thua Thien Hue Hue, Vietnam. After being collected, waterhyacinth would be dried for 5-7 days and used as raw material for the process of organic fertilizer production.

Isolation of cellulolytic fungi

The potato glucose agar (PGA) medium was used for the isolation of filamentous fungi. To isolate the fungi, 1 g of soil sample was suspended in 9 ml of sterile distilled water and vortexed thoroughly. From this 10 ml stock solution, serial dilutions were performed to 10^{-6}. 100 µl from the dilutions of 10^{-5} was plated in triplicates on the culture medium. Cultures were then incubated at 30 ^{o}C. The growths of fungi were daily checked. Pure isolates were maintained at 4 ^{o}C in a refrigerator for further analyses (Akharaiyi et al., 2016).

Determination of cellulase activity

25 ml of medium, given 2% agar and 1% carboxymethylcellulose, was poured into each petri dish. Next, fungus isolates were subcultured on the medium. Cultures were then incubated at 30 ^{o}C in the dark. After 4 days of incubation, 5 ml of Lugol's reagent was plated on each petri dish to detect clear zones. Finally, the diameters of clear zones were obtained (Coronado-Ruiz et al., 2018).

Effect of pH and temperature on cellulase activity

The activity of cellulase was measured by changing the pH with 0.05 M sodium phosphate buffer. The pH was varied between 5.0-7.5, after which cellulase activity was determined. The effect of temperature on cellulase activity was investigated at different temperatures of range 30-70 ^{o}C with pH being adjusted based on cellulase activity determined during pH studies (Talekar et al., 2011).

Effect of culture duration

To determine the effect of culture duration on cellulase activity, the filamentous fungi was grown on rice bran medium supplemented with carboxymethylcellulose substrate and incubated at 30 ^{o}C. After 24-168 hours of incubation, the enzyme activity would be determined based on the diameters of clear zone (Bansal et al., 2012).

Classification of filamentous fungi

Isolates were firstly pre-classified by observing morphological features of colonies on agar plates and reproductive organs on glass slide at 40X magnification (Klich et al., 2002).

The D1/D2 region of 28S rRNA coding gene was amplified using the primer pair U1/U2 (U1FGC (5 '- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GTG AAA TTG TTG AAA GGG AA - 3'; Sigma) and U2R (5 '- GAC TCC TTG GTC CGT GTT - 3'; Sigma)). 260 bp amplicons were then sequenced by Nam Khoa Trade Production & Service Company Limited, Vietnam. After that, the isolate N.S8 was classified using BLASTN, with the sequenced segment as query sequence (Sandhu et al., 1995).

Compost preparation

The filamentous fungi with high cellulase activity was cultured in 250 ml Erlenmeyer flasks containing 1% of carboxymethylcellulose and rice bran. During the culture process, the humidity would be maintained appropriately (Tolan et al., 1999). The experiment of composting water hyacinth consisted of two treatments which are presented in Table 1.

Table 1. Treatments for composting water hyacinth

Treatment	Water hyacinth (kg)	Cellulolytic microorganism	Concentration (%)
Experiment	100	Strain with the highest capacity of degrading cellulose	5
Control	100	-	-

100 kg of dried water hyacinth and 5 kg of fungus were well mixed. The mixture was then compacted, covered by polyethylene sheets and incubated for 45 days. Control experiment was carried out in the same way as above-mentioned without the addition of fungus.

After 45 days of incubation, the total nitrogen (N), phosphorus (P_2O_5) and potassium (K_2O) of the compost were determined by ISO 11261:1995, wet digestion spectrophotometer (420 nm) and flame photometer respectively (Barton, 1948; Hesse & Hesse, 1971).

Data analysis

The data were analyzed by ANOVA using IBM® SPSS® Statistic software (version 20). Means were compared by Duncan's test with the significance level of

0.05. All the graphs were constructed by Microsoft® Office Excel software (2013). Each experiment was done in triplicates.

Results and Discussion

Isolation and screening of cellulolytic fungi

Thirteen filamentous fungi were isolated from different soil sources. The isolates were screened and compared for their ability of degrading cellulose. Isolates' mean clear zone diameters, obtained after 4 days of incubation, are presented in Table 2.

Table 2. Growth and enzyme activity of isolates

Isolate's code	Colony diameter (mm)	Clear zone diameter (mm)
N.S1	17(0.421)^{f*}	17(0.234)^{g}
N.S2	28(0.528)^{d}	28(0.442)^{d}
N.S3	19(0.526)^{e}	19(0.521)^{f}
N.S4	16(0.415)^{f}	16(0.218)^{g}
N.S5	17(0.432)^{f}	19(0.322)^{f}
N.S6	42(0.846)^{b}	42(0.865)^{c}
N.S7	13(0.408)^{g}	17(0.831)^{g}
N.S8	**45(1.52)^{a}**	**60(0.963)^{a}**
N.S9	35(0.835)^{c}	45(0.958)^{b}
N.S10	9(0.405)^{h}	16(0.524)^{h}
N.S11	15(0.524)^{g}	15(0.519)^{h}
N.S12	14(0.423)^{g}	22(0.633)^{e}
N.S13	15(0.432)^{g}	23(0.631)^{e}

*Within a column, means having a letter in common are not significantly different at the 5% level. The values are presented as mean (standard deviation).

The insignificant differences between colonies and clear zones' diameters were observed in 6 isolates namely N.S1, N.S2, N.S3, N.S4, N.S6 and N.S11. In contrast, isolates including N.S5, N.S7, N.S8, N.S9, N.S10 N.S12 and N.S13 obviously showed high cellulase activity, with the largest clear zones' diameters, 60 mm on average, created by N.S8. Thus, among 13 isolated fungi, 7 isolates were identified as cellulase producers. Additionally, because of the highest cellulase activity, isolate N.S8 was selected to use for further studies.

Morphological characterization of the isolate N.S8

Morphological examination showed that N.S8's colonies were initially white and then turned to the yellow color of areca flower as cultures aged. The surfaces of colonies were velvety, with clearly observed hyphae. From the microscopic observation, it is clear that the hyphae, containing septa, are olive green and branched. The conidiophore is not branched. Conidia are round and rough (Figure 1).

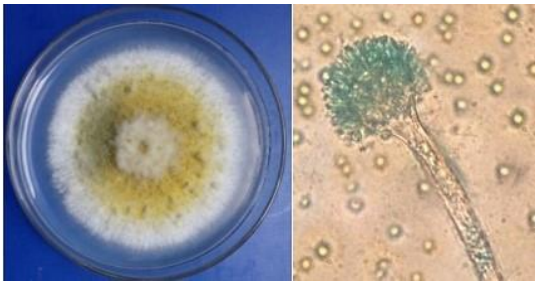

Figure 1. Features of isolate N.S8.

Identification on the basis of phylogenetic analyses

The results show that the sequenced D1/D2 region of NS.8 is highly similar to the one of *A. oryzae* (Accession number: AP007172.1), with the identity value and E-value of 100% and 0.0 respectively. Thus, it can be concluded that NS.8 belongs to *A. oryzae* species (Table 3 and Table 4).

Table 3. Evaluating homologous rate of 28S RNA gene sequence

Species name	Strain name	Accession number	Homologous rate (%)
Aspergillus oryzae	RIB40	AP007172.1	100

Table 4. The sequencing of the 28S rRNA gene of the isolated strain

Query	1	CACGGGCGCGGACACCCCATCCCAGACGGGATTCTCACCCTCTCTGACGGCCCGTTCCAG	60
Sbjct	3119	CACGGGCGCGGACACCCCATCCCAGACGGGATTCTCACCCTCTCTGACGGCCCGTTCCAG	3060
Query	61	GGCACTTAGACAGGGGCCGCACCCGAAGCATCCTCTGCAAATTACAATGCGGACCCCGAA	120
Sbjct	3059	GGCACTTAGACAGGGGCCGCACCCGAAGCATCCTCTGCAAATTACAATGCGGACCCCGAA	3000
Query	121	GGAGCCAGCTTTCAAATTTGAGCTCTTGCCGCTTCACTCGCCGTTACTGAGGCAATCCCG	180
Sbjct	2999	GGAGCCAGCTTTCAAATTTGAGCTCTTGCCGCTTCACTCGCCGTTACTGAGGCAATCCCG	2940
Query	181	GTTGGTTTCTTTTCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTATCCCTACCTGAT	240
Sbjct	2939	GTTGGTTTCTTTTCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTATCCCTACCTGAT	2880
Query	241	CCGAGGTCAACCTGGAAAAAGATTGATTTGCGTTCGGCAAGCGCCGGCCGGGCCTACAGA	300
Sbjct	2879	CCGAGGTCAACCTGGAAAAAGATTGATTTGCGTTCGGCAAGCGCCGGCCGGGCCTACAGA	2820
Query	301	GCGGGTGACAAAGCCCCATACGCTCGAGGATCGGACGCGGTGCCGCCGCTGCCTTTGGGG	360
Sbjct	2819	GCGGGTGACAAAGCCCCATACGCTCGAGGATCGGACGCGGTGCCGCCGCTGCCTTTGGGG	2760
Query	361	CCCGTccccccGGAGAGGGGACGACGACCCAACACACAAGCCGTGCTTGATGGGCAGCA	420
Sbjct	2759	CCCGTCCCCCCCGGAGAGGGGACGACGACCCAACACACAAGCCGTGCTTGATGGGCAGCA	2700
Query	421	ATGACGCTCGGACAGGCATGCCCCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGAC	480
Sbjct	2699	ATGACGCTCGGACAGGCATGCCCCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGAC	2640
Query	481	TCGATGATTCACGGAATTCTGCAATTCACACTAGTTATCGCATTTCGCTGCGTTCTTCAT	540
Sbjct	2639	TCGATGATTCACGGAATTCTGCAATTCACACTAGTTATCGCATTTCGCTGCGTTCTTCAT	2580
Query	541	CGATGCCGGAACCAAGAGATCCATTGTTGAAAGTTTTAACTGATTGCGATACAATCAACT	600
Sbjct	2579	CGATGCCGGAACCAAGAGATCCATTGTTGAAAGTTTTAACTGATTGCGATACAATCAACT	2520
Query	601	CAGACTTCACTAGATCAGACAGAGTTCGTGGTGTCTCCGGCGGGCGCGGGCCCGGGGCTG	660
Sbjct	2519	CAGACTTCACTAGATCAGACAGAGTTCGTGGTGTCTCCGGCGGGCGCGGGCCCGGGGCTG	2460
Query	661	AGAGCCCCCGGCGGCCATGAATGGCGGGCCCGCCGAAGCAACTAAGGTACAGTAAACACG	720

Determination of the suitable conditions for cellulase synthesis

Effect of culture duration

It is illustrated in Figure 2 that when the culture duration was increased, the cellulase activity of *A. oryzae* rose. However, when the optimal culture duration was exceeded, the enzyme activity began to drop. The maximum cellulase activity was attained after 144 h of incubation with a clear zone diameter of 38.67 mm on average.

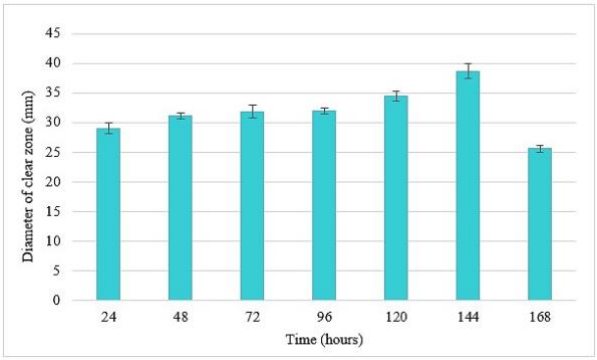

Figure 2. Effect of time on *A. oryzae* cellulase activity. Error bars indicate standard deviation.

Compared to the previous research, differences were observed in the results of our present study. Specifically, according to Acharya et al. (2008), the highest cellulase activity was obtained after 96 hours of fermentation using *A. niger* (Acharya et al., 2008). Additionally, another research on cellulase production by *A. niger* from coastal mangrove debris conducted by Devanathan et al. (2007) also indicated that the optimal fermentation duration is 96 hours (Devanathan et al., 2007). Thus, all the above-mentioned results proved that the culture duration can affect the ability of producing cellulase of the filamentous fungi. Moreover, the optimal culture duration and enzyme amount are different among fungus strains (Nochure et al., 1993).

Effect of pH

As it is highlighted in Figure 3, the highest cellulase activity was obtained at pH 6.5 with the average clear zone diameter of 37.55 mm. According to Anita et al. (2009), the optimal pH for cellulase activity produced by *A. heteromorphus* is 6.0 (Anita et al. 2009). Furthermore, in a study conducted by Akiba et al. (1995), the authors concluded that the optimal pH for cellulase activity from *A. niger* is 6.0-7.0, and the stable pH range is 5.0-10.0 (Akiba et al., 1995). These different results might be caused by the genetic differences within the same genus (Acharya et al., 2008).

Effect of temperature

The effect of temperature on cellulase activity was determined in the range of 30-70 °C. As illustrated in Figure 4, the optimum temperature was 40 °C. Beyond 40 °C, thermostability decreased, possibly due to thermal denaturation of enzyme. The results obtained from this study are similar to the findings of Ali et al. (1991) who concluded that the optimal temperature for cellulase activity from *A. niger* "Z10" and *A. terreus* was at 40 °C. Loss of cellulase activity was observed beyond 40 °C and 50 °C (Ali et al., 1991).

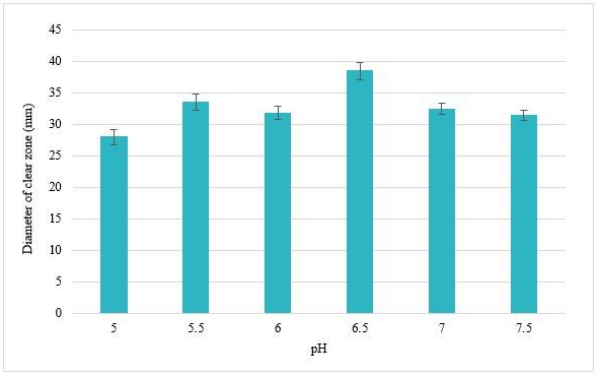

Figure 3. Effect of pH on *A. oryzae* cellulase activity. Error bars indicate standard deviation.

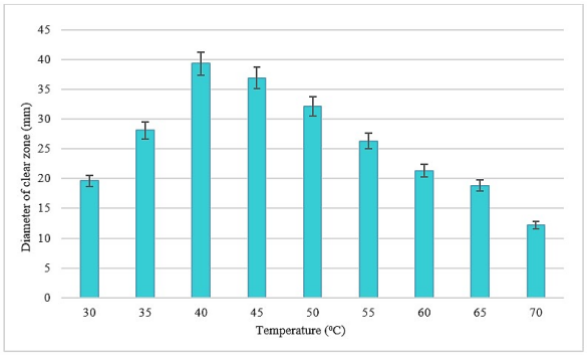

Figure 4. Effect of temperature on *A. oryzae* cellulase activity. Error bars indicate standard deviation.

Creating organic fertilizer from water hyacinth and *A. oryzae* "N.S8"

After 6 days of culturing the fungus in the optimized condition, 100 kg of dried water hyacinth and 5 kg *A. oryzae* were mixed. After 45 days of incubation, chemical parameters of the organic fertilizer were shown in Table 3.

The provided results in Table 5 indicated that after being incubated, the total N, P, and K contents in all treatments increased. However, the contents of N, P, K in the treatment containing microorganisms was higher than those of the control with 1.53%, 0.19% and 0.38%, respectively.

The nutrient compositions of organic fertilizer from water hyacinth in the present research are higher compared to that in the previous research of Thanaporn et al. (2019) who concluded that the ranges of chemical parameters in the liquid organic fertilizer produced from agricultural residues and industrial wastes are 0.14-0.33% (total N), 0.002-0.017% (total P_2O_5) and 0.81-11.8% (total K_2O) (Thanaporn et al., 2019).

Table 5. Analysis results of total N, P, K contents in organic fertilizer sample

Content (%)	Before incubation		After incubation	
	Control	Experimental treatment (Supplemented with 5% *A. oryzae* "N.S8")	Control	Experimental treatment (Supplemented with 5% *A. oryzae* "N.S8")
Total N	1.42	1.42	1.53	3.35
Total P	0.16	0.16	0.19	0.43
Total K	0.36	0.36	0.38	0.74

Conclusion

Thirteen filamentous fungi with the ability of degrading cellulose were isolated from different soil sources in which the isolate N.S8 showed the highest cellulase activity with the average diameter of clear zone of 35 mm. The results of classification indicated that this isolate is *A. oryzae*.

The optimal culture duration and pH for growth and development of *A. oryzae* "N.S8" in rice bran medium are 144 hours and pH 6.5.

The use of *A. oryzae* "N.S8" for composting water hyacinth created an organic fertilizer with nitrogen (N), phosphorus (P_2O_5), and potassium (K_2O) contents of 3.35%, 0.43% and 0.74%, respectively after 45 days.

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BIOTECH STUDIES

RESEARCH PAPER

Role of grafting in tolerance to salt stress in melon (*Cucumis melo* L.) plants: ion regulation and antioxidant defense systems

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Grafting
Ion accumulation
Rootstock
Salinity

Abstract

Grafting in vegetables is a method that has been commonly used in recent years, not just for the treatment of soil borne diseases and pests, but also to facilitate higher abiotic stress tolerance under conditions such as salinity. Herein, it was aimed to determine if the salt tolerance of two salt-susceptible melon genotypes, SCP-1 and SCP-2, could be improved by grafting onto TLR-1 and TLR-2, which are salt-tolerant melon genotypes, and Albatros commercial melon rootstock. The grafted plants were grown in plastic pots containing a peat: perlite mixture and exposed to NaCl at doses of 0 and 200 mM under greenhouse conditions. The salt-tolerant rootstock significantly diminished the damaging effects caused by salt stress via a reduction in the uptake of Na and Cl, which enhanced Ca and K uptake and micronutrition. Stress-induced activities of catalase, superoxide dismutase, ascorbate peroxidase, and glutathione reductase were considerably higher in the grafted plants. The results showed that grafting salt susceptible plants onto the salt-tolerant rootstock improved the growth regulation. The salt tolerance of the grafted melon seedlings may have partially been the result of the decreased Na and Cl, and malondialdehyde contents and higher antioxidant enzyme activities.

Introduction

Salinity is one of the most crucial abiotic conditions and the cause of reduced plant growth, development, and production worldwide, especially in arid and semiarid areas, in which there is insufficient precipitation, and the soils contain high concentrations of salt. Salt stress paves the way for morphological, physiological, and biochemical response changes in plants. Salinity is pertinent to many conditions, such as an imbalance in nutrition, changes in processes of metabolism, and cell and chloroplast membrane distortion (Sarabi et al., 2017). Sodium (Na) is an important cation that is soluble in a lot of soils in arid and semiarid areas. When there is a concentrated amount of Na in the soils, harmful effects can be noticed in plants. One of the essential effects of salinity is a nutritional imbalance. The high amount of Na and chlorine (Cl) accumulation under salt stress is regarded as destructive to the cellular systems of plants, and not only calcium (Ca) and potassium (K) ions are damaged, but Na homeostasis also takes place. In order to endure salt stress, plants tend to decrease Na loading into their xylem, thus limiting the accumulation of Na in their tissues (Tavakkoli et al., 2011). Abiotic stressors such as salinity cause an excessive accumulation of reactive oxygen species (ROS), which could result in protein oxidation, lipid peroxidation, enzyme inactivation, damage to DNA, and/or interaction with other essential plant cell components. To minimize the toxic effects caused by ROS, plants possess various kinds of enzymatic and nonenzymatic antioxidative systems. The enzymatic system comprises superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR), while nonenzymatic antioxidants comprise reduced glutathione (GSH),

ascorbate, carotenoids, and tocopherol (Kusvuran et al., 2016).

Over recent decades, there have been several strategies proposed and used to minimize the effects of salt stress on productivity in the field of agriculture, with the aim of facilitating the development and use of highly saline soils. Reclamation of saline soil and soil salinity correction are important agricultural strategies; however, they are temporary and fairly costly. Breeding of resistant genotypes has also been a method used to aid crops such as horticultural crops in improving their resistance to salt stress. However, it is a laborious and complex process because of the polygenic nature of a plant's resistance to salt. As a result of these complexities, using genotypes that are resistant to salinity as a rootstock has been considered a simple and efficient way of improving a crop's resistance to salt stress (Yanyan et al., 2018). Accordingly, discovering new ways to produce plants that are more resilient to effects caused by salt stress remains one of the highest priority objectives. The grafting of genotypes that are high yielding onto salt-tolerant rootstocks is one method that can be used to achieve this objective (Santa-Cruz et al., 2002). It has been estimated that the grafting of rootstock might induce a change in the balance of the hormones in scions, as it causes alterations in the characteristics of the scion, such as plant hydraulics, and the uptake of water and nutrients, which are affected by vascular connection disruptions (Fullana-Pericas et al., 2018). As a result of this, grafting is a widely applied method to improve the salt tolerance of plants by placing a scion that is productive onto a tolerant rootstock, to initiate tolerance against stress by physical means (Simpson et al., 2015). Grafting is considered to be a vital approach that alleviates the consequences of abiotic stress factors, particularly in tomato and melon (Penella et al., 2016). Melon (*Cucumis melo* L.), of the family Cucurbitaceae, is a significant horticultural crop that is mainly grown in arid and semiarid regions where salt stress remains a serious problem, despite the advanced management techniques that have been developed over the past decades (Ulas et al., 2019).

In the current study, the effects of grafting on the physiological and biochemical responses of plants under salt stress, with salt-tolerant melon genotypes, 'TLR-1 and TLR-2', and Albatros as the rootstocks and salt-susceptible genotypes, SCP-1 and SCP-2, as the scions were investigated. The study evaluated the salt sensitivity of melon seedlings that had been grafted with a rootstock that was salt-tolerant and provided the groundwork for the improvement of salt tolerance via grafting. Furthermore, the relation between the grafting and salt tolerance improvement was determined. Therefore, an investigation of the effectiveness of grafting utilization in order to increase tolerance to salinity in melon, which is an important species in arid and semiarid regions, was conducted herein.

Materials and Methods

Plant materials and growth conditions

Three rootstocks, comprising TLR-1 and TLR-2, which were the selected salt-tolerant melon genotypes in the previous study of Kusvuran (2012), and Albatros (*Cucumis melo*), (Rito Seeds Company, Turkey), which is a commercial rootstock, were used. As the scion, SCP-1 and SCP-2, which are salt-sensitive melon genotypes, were used. All of the rootstocks were grafted with the SCP-1 and SCP-2 scions, and therefore the following graft combinations were obtained: SCP-1/TLR-1, SCP-1/TLR-2, SCP-2/TLR-1, SCP-2/TLR-2, SCP-1/Albatros, and SCP-2/Albatros. The control treatment comprised of the 4 genotypes, *C. melo* (TLR-1, TLR-2, SCP-1, and SCP-2) and a commercial variety (Albatros), which were not grafted and allowed to develop their own roots. Grafting was performed according to the one cotyledon method presented by Lee in 1994. Both the grafted and ungrafted plants were repotted into 12-L plastic pots that contained a peat: perlite mixture at a ratio of 2:1. The plants were then maintained under greenhouse conditions which comprised day/night temperatures of 26 ± 2 °C and 18 ± 2 °C, with a relative humidity of 65% ± 5. Each of the pots contained 4 plants, and each experiment was done with 4 replications. In this experiment, the administration of salinity was initialized 13 days after transplanting. On the first day of experiment, the plants were treated with 50 mM NaCl, and the dose was increased by 50 mM NaCl each day until the day 4, to a total of 200 mM NaCl. In this study, the treated (experimental) and untreated (control) grafted and ungrafted melon plants were all grown for 14 days. Upon conclusion of the experiment, evaluation of the plants was performed via morphological, physiological, and biochemical parameters.

Relative water content (RWC) and leaf water potential (Ψw): Estimation of the RWC was performed using the method of Turkan et al. (2005). Calculation of the RWC was performed as follows: RWC (%) = [(FW – DW) / (TW – DW)] × 100. Here, TW represents the turgid weight after floatation on deionized water for 5 h. The 3rd fully expanded leaf was measured across the middle of the photoperiod to attain the leaf Ψw via a pressure chamber (Muries et al., 2013).

Ion concentration

For the determination of ion contents, the melon leaves and roots were dried for 48 h at 65 °C. After which, a mill with a 20-mesh sieve was used to grind the samples. The leaf powder was turned into ash for about 6 h at 550 °C, which was then dissolved in 3.3% HCl. Atomic absorption spectrometry was used to determine the Na, K, Ca, Fe, Mn, Zn, Cu concentrations in the roots and leaves. The Cl concentration in the tissue samples were determined using the titrimetric analysis with silver nitrate ($AgNO_3$) via the Mohr method (Dasgan et al., 2018).

Malondialdehyde (MDA) content

Measurement of the lipid peroxidation was calculated using the MDA level determined by the thiobarbituric acid (TBA) reaction (Heath & Packer, 1968) of the supernatant at 10,000 × *g* at 4 °C for 10 min, the absorbance was read at 532 nm and values were subtracted if they corresponded to non-specific absorption at 600 nm.

Photosynthetic pigments

The method suggested by Arnon (1949) was used to determine the total carotenoid, chlorophyll a (Chla), and chlorophyll b (Chlb) contents. The leaf pigment extracted was performed with 80% (v/v) acetone and a Shimadzu UV mini-1240 spectrophotometer (Kyoto, Japan) was used to measure the absorbance of the extraction at 663, 645, and 470 nm.

Antioxidant enzyme activities

A mortar and pestle were used for extraction of the enzymes from 0.5 g of the leaf tissue, in addition to 5 mL of extraction buffer that contained 50 mM of potassium-phosphate buffer, at pH 7.6, and 0.1 mM of disodium ethylenediaminetetraacetate. Centrifugation of the homogenate took place for 15 min at 15,000 × *g*, and the supernatant fraction was used to perform the enzyme assay. The preparation operations for the enzyme extraction were all done at 4 °C. The SOD assay was performed according to the method of Cakmak & Marschner (1992), via monitoring the reduction of the superoxide radical (O_2^-)-induced nitro blue tetrazolium at 560 nm. Monitoring of the disappearance of hydrogen peroxide was used for the determination of the CAT activity. Measurement of the ascorbate consumption from its absorbance at 290 nm was performed to determine the APX activity. The amount of enzyme required to consume 1 µmol of ascorbate min^{-1} was determined as 1 unit of APX activity (Cakmak & Marschner, 1992). From the absorbance of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm, measurement of its enzyme-dependent oxidation was used to determine the GR activity. The volume of the enzyme that oxidized 1 µmol of NADPH min^{-1} was defined as 1 unit of GR activity.

Statistical analysis

The plot design used for the experiment was completely randomized, comprising 4 replicates. The mean values were compared using the Tukey multiple range test. Statistical significance was accepted as $P< 0.05$ using SPSS v.13.0 software for Windows (SPSS Inc., Chicago, IL, USA). Data were presented as the mean ± standard deviation (SD) and error bars represent the standard errors of the means in all of the figures.

Results

According to the findings of the present study, salt stress conditions inhibited plant development and growth, but these effects varied between combinations, grafting, and non-grafting (Table 1). The growth of the ungrafted melon seedlings was inhibited significantly following the application of NaCl, while the effects of salt stress were less severe in the grafted seedlings. Under salt stress, the fresh and dry weights of the shoot and root, as well as the leaf area, of ungrafted seedlings were reduced by 70%, 55%, 48%, 43%, and 82%, respectively, whereas they were reduced by 33%, 24%, 26%, 29%, and 26%, respectively, in grafted plants.

In addition, all of the plants that received an application of 200 mM NaCl, SCP-1/TLR-2, SCP-2/TLR-2, SCP-1/Albatros, and SCP-2/Albatros, exhibited growth parameters with the least decrease of all of the grafted combinations.

As a result of the grafting and salt application interaction, the RWC and leaf Ψw showed significant difference at $P< 0.001$ level. Melon seedlings under salt stress showed decreased RWC at 31% and it was 33% in ungrafted plants when compared to the controls (Table 1). However, in the grafted plants, significantly improved RWC was observed at rates of 82% and 123% when compared to the salt-stressed ungrafted plants. With the application of 200 mM NaCl, maximum improvement in the RWC was observed with the

Table 1. Effects of rootstock on the growth parameters of the melon seedlings under salt stress

Graft Combination	T	Shoot fresh weight (g plant^{-1})	Shoot dry weight (g plant^{-1})	Root fresh weight (g plant^{-1})	Root dry weight (g plant^{-1})	Leaf area (cm^2 plant^{-1})	RWC (%)	Leaf Ψw
SCP-1	C	129.21 ± 10.59^{h}	14.25 ± 3.56$^{d–f}$	3.21 ± 0.09^{fg}	0.30 ± 0.09$^{g–i}$	2977.82 ± 120.44$^{d–f}$	80.00 ± 6.88$^{a–c}$	–0.24 ± 0.09^{ab}
	S	60.16 ± 1.41^{j}	8.15 ± 2.03^{fg}	1.97 ± 0.37^{h}	0.18 ± 0.04^{i}	403.79 ± 34.83^{h}	31.95 ± 7.74^{e}	–0.59 ± 0.04^{gh}
SCP-1/TLR-1	C	136.67 ± 4.01^{gh}	15.24 ± 1.13$^{c–f}$	4.11 ± 0.54$^{d–f}$	0.34 ± 0.05$^{g–i}$	2627.29 ± 120.04^{f}	81.94 ± 7.27^{ab}	–0.22 ± 0.03^{a}
	S	74.87 ± 4.33^{j}	10.68 ± 0.49$^{e–g}$	3.18 ± 0.07^{fg}	0.25 ± 0.03^{hi}	1916.19 ± 139.44^{g}	61.21 ± 3.37^{d}	–0.48 ± 0.04^{ef}
SCP-1/TLR-2	C	170.42±10.86$^{c–e}$	20.35 ± 1.50$^{b–d}$	4.35 ± 0.59$^{c–f}$	0.40 ± 0.05^{gh}	3863.51 ± 198.22^{ab}	83.69 ± 5.78^{ab}	–0.27 ± 0.04$^{a–c}$
	S	131.79 ± 8.38^{h}	17.04 ± 0.72$^{b–d}$	3.67 ± 0.25^{ef}	0.35 ± 0.06$^{g–i}$	3198.09 ± 345.26$^{c–f}$	71.42 ± 5.78$^{b–d}$	–0.37 ± 0.04^{cd}
SCP-1/Albatros	C	188.74 ± 8.27^{bc}	23.02 ± 2.92^{ab}	4.44 ± 0.44$^{c–f}$	0.49 ± 0.10^{fg}	3479.10 ± 309.52$^{b–e}$	81.33 ± 5.38^{ab}	–0.21 ± 0.03^{a}
	S	141.94 ± 6.97$^{f–h}$	18.11 ± 2.92$^{b–d}$	3.58 ± 0.16^{ef}	0.40 ± 0.05^{gh}	2917.19 ± 156.93^{ef}	70.30 ± 6.43$^{b–d}$	–0.33 ± 0.03$^{b–d}$
SCP-2	C	177.71 ± 5.11^{cd}	18.15 ± 2.45$^{b–d}$	4.74 ± 0.60$^{c–e}$	0.62 ± 0.08^{ef}	3588.38 ± 384.52$^{a–d}$	91.38 ± 4.19^{a}	–0.27 ± 0.04$^{a–c}$
	S	60.63 ± 6.95^{j}	7.79 ± 1.38^{g}	2.33 ± 0.12^{gh}	0.36 ± 0.07$^{g–i}$	982.80 ± 88.40^{h}	33.13 ± 4.13^{e}	–0.64 ± 0.05^{h}
SCP-2/TLR-1	C	158.77 ± 8.65$^{d–f}$	19.11 ± 4.59$^{b–d}$	5.38 ± 0.66^{bc}	0.94 ± 0.16^{c}	3899.50 ± 407.65^{ab}	82.06 ± 3.48^{ab}	–0.23 ± 0.03^{a}
	S	104.85 ± 8.65^{i}	14.36 ± 1.05$^{d–f}$	4.07 ± 0.30$^{d–f}$	0.66 ± 0.04$^{d–f}$	2710.55 ± 157.91^{f}	60.34 ± 8.46^{d}	–0.52 ± 0.02^{fg}
SCP-2/TLR-2	C	208.81 ± 9.57^{ab}	21.14 ± 2.94$^{a–c}$	6.10 ± 0.46^{b}	0.87 ± 0.15^{cd}	4173 ± 304.59^{a}	84.28 ± 4.77^{ab}	–0.23 ± 0.04^{a}
	S	159.36 ± 5.74$^{d–f}$	17.61 ± 0.87$^{b–d}$	5.05 ± 0.59$^{b–d}$	0.72 ± 0.07^{de}	3649.86 ± 274.01$^{a–c}$	64.58 ± 3.12^{cd}	–0.42 ± 0.03$^{d–f}$
SCP-2/Albatros	C	215.65 ± 10.73^{a}	26.94 ± 4.77^{a}	7.61 ± 0.85^{a}	1.55 ± 0.11^{a}	3945.19 ± 233.16^{ab}	81.84 ± 7.64^{ab}	–0.22 ± 0.03^{a}
	S	153.94 ± 5.78$^{e–g}$	19.27 ± 1.06$^{b–d}$	6.22 ± 0.39^{b}	1.16 ± 0.06^{b}	3048.54 ± 372.61$^{c–f}$	68.83 ± 6.07$^{b–d}$	–0.39 ± 0.02^{de}

*Each value represents the mean of four replicates. For each parameter, different superscripted letters represent statistically significant differences at $P\leq0.05$ according to the Tukey test (T: treatment, S: salinity, C: control).

Albatros rootstock combination for both SCP-1 and SCP-2, where the increase in RWC was 120% and 127%. The leaf Ψw ranged from –0.21 to –0.64 MPa and was significantly affected by the salt stress. These results showed that grafting promoted a positive influence on the Ψw under salt stress (increase of 18% and 44%).

Salt stress generally increased the levels of Na and Cl in the tissue samples, with the grafted plants showing a lower concentration in the leaves (decreased by 24% and 52%, respectively) and roots (decreased by 10% and 71%, respectively) when compared with the ungrafted plants (Figure 1). Although exposure to NaCl also elevated the leaf K levels of the grafted plants (increased by 48% and 135%), the roots of these plants had lower K concentrations (decreased by 5%–15% more than the roots of the ungrafted plants. Similarly, Ca levels were higher in the leaves (increased by 38% and 135%) of the grafted plants than in the corresponding samples from the ungrafted plants. Finally, concentrations of micro-nutrients, such as Fe, Cu, Mn, and Zn, in the leaves and roots were significantly affected by the melon rootstock, at 12% and 156% higher for Fe, 27% and 187% higher for Mn, and 4% and 56% higher for Zn, when compared with the ungrafted salt-stressed plants, whereas the Cu contents were decreased by an average of 11% in the leaves (Table 2).

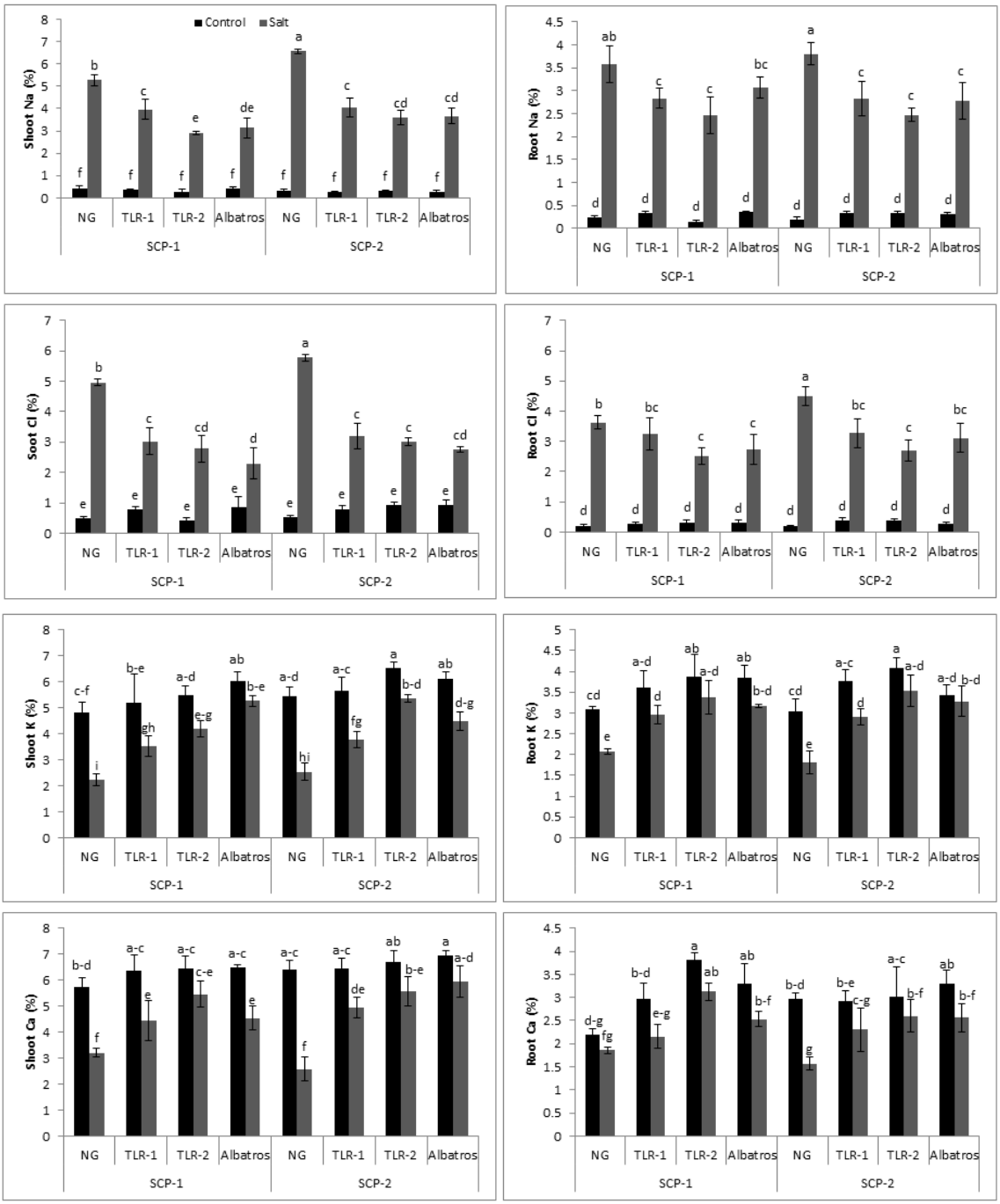

Figure 1. Effects of rootstock on the Na^+ (A, B), Cl^- (C, D), K^+ (E, F), and Ca^{2+} (G, H) contents of the melon seedlings under salt stress. Each value represents the mean of four replicates. For each parameter, different letters represent statistically significant differences at $P < 0.05$ according to the Tukey test.

Table 2. Effects of rootstock on the micronutrient contents of the melon seedlings under salt stress

Graft Combination	T	Fe (ppm)		Cu (ppm)		Mn (ppm)		Zn (ppm)	
		Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
SCP-1	C	70.90 ± 9.85^{ef}	94.45 ± 9.63^{c-f}	43.85 ± 5.69^{a}	4.93 ± 1.46^{ef}	41.92 ± 3.97^{b-f}	40.54 ± 3.61^{f}	40.65 ± 6.79^{fg}	42.53 ± 3.78^{g-i}
	S	50.97 ± 8.90^{f}	42.20 ± 2.65^{j}	28.75 ± 4.89^{b-d}	2.10 ± 0.45^{f}	27.25 ± 5.94^{fg}	29.61 ± 2.60^{g}	19.98 ± 2.53^{i}	37.48 ± 0.70^{hi}
SCP-1/TLR-1	C	78.95 ± 7.51^{de}	78.85 ± 4.49^{fg}	15.50 ± 5.88^{ef}	12.38 ± 1.36^{ab}	45.38 ± 6.68^{a-e}	57.05 ± 2.74^{cd}	47.60 ± 5.27^{d-f}	44.93 ± 5.23^{f-h}
	S	64.70 ± 6.39^{ef}	59.68 ± 4.59^{hi}	14.00 ± 2.83^{ef}	7.65 ± 1.68^{c-e}	39.46 ± 6.91^{c-g}	48.43 ± 3.77^{d-f}	32.83 ± 2.63^{gh}	40.50 ± 1.91^{g-i}
SCP-1/TLR-2	C	149.63 ± 9.79^{a}	104.75 ± 4.83^{b-d}	15.25 ± 3.99^{ef}	11.50 ± 1.91^{a-c}	51.87 ± 3.36^{a-c}	65.91 ± 2.56^{b}	70.08 ± 5.45^{a}	45.45 ± 2.77^{e-h}
	S	130.80 ± 6.41^{ab}	83.40 ± 6.00^{e-g}	24.72 ± 4.23^{c-f}	9.23 ± 1.33^{b-d}	46.92 ± 6.66^{a-d}	56.18 ± 2.74^{cd}	58.38 ± 3.73^{b-d}	40.93 ± 5.10^{g-i}
SCP-1/Albatros	C	109.53 ± 4.53^{bc}	113.40 ± 3.27^{b}	23.69 ± 5.39^{c-f}	9.35 ± 2.01^{b-d}	58.51 ± 3.82^{a}	64.25 ± 3.18^{bc}	58.85 ± 3.28^{a-d}	53.55 ± 3.80^{c-e}
	S	87.63 ± 10.12^{c-e}	91.93 ± 4.52^{d-f}	31.62 ± 6.50^{a-d}	11.50 ± 1.91^{a-c}	57.16 ± 8.09^{ab}	64.54 ± 2.28^{bc}	46.45 ± 7.30^{ef}	52.20 ± 2.43^{c-f}
SCP-2	C	88.83 ± 6.18^{c-e}	108.90 ± 8.21^{b-d}	39.50 ± 6.24^{ab}	6.70 ± 1.79^{de}	33.25 ± 4.42^{d-g}	49.53 ± 3.17^{d-f}	54.98 ± 3.46^{b-e}	44.55 ± 3.68^{f-h}
	S	50.43 ± 4.95^{f}	54.30 ± 9.44^{ij}	26.25 ± 4.27^{c-e}	1.65 ± 0.04^{f}	23.29 ± 3.79^{g}	26.48 ± 3.13^{g}	28.80 ± 4.13^{hi}	34.30 ± 1.27^{i}
SCP-2/TLR-1	C	104.95 ± 10.58^{b-d}	71.73 ± 8.39^{gh}	24.50 ± 4.18^{c-f}	9.00 ± 1.66^{b-e}	48.48 ± 5.20^{a-d}	52.75 ± 3.09^{de}	56.95 ± 7.30^{b-e}	54.18 ± 3.17^{cd}
	S	80.43 ± 13.76^{de}	61.33 ± 7.91^{hi}	22.25 ± 6.34^{c-f}	5.60 ± 1.74^{d-f}	29.41 ± 5.56^{e-g}	45.92 ± 3.67	41.43 ± 3.70^{fg}	47.43 ± 2.88^{d-g}
SCP-2/TLR-2	C	109.95 ± 4.13^{bc}	97.38 ± 5.29^{b-e}	12.25 ± 3.09^{f}	7.80 ± 1.63^{c-e}	51.59 ± 8.98^{a-c}	76.65 ± 2.66^{a}	64.48 ± 4.05^{ab}	57.43 ± 2.36^{c}
	S	98.40 ± 6.62^{cd}	82.33 ± 6.88^{e-g}	18.92 ± 5.93^{d-f}	8.83 ± 1.27^{b-e}	44.74 ± 3.99^{a-e}	76.18 ± 3.26^{a}	53.03 ± 3.53^{c-e}	51.53 ± 2.41^{c-f}
SCP-2/Albatros	C	100.68 ± 2.70^{cd}	132.91 ± 2.35^{a}	26.41 ± 3.44^{c-e}	7.62 ± 1.79^{c-e}	54.27 ± 4.92^{a-c}	63.20 ± 2.69^{bc}	61.93 ± 5.89^{a-c}	77.45 ± 2.94^{a}
	S	90.15 ± 13.65^{c-e}	111.88 ± 8.27^{b}	35.23 ± 4.12^{a-c}	14.05 ± 1.43^{a}	49.70 ± 2.49^{a-d}	50.70 ± 7.49^{de}	51.53 ± 0.85^{c-f}	66.50 ± 4.24^{b}

*Each value represents the mean of four replicates. For each parameter, different superscripted letters represent statistically significant differences at *P< 0.05* according to the Tukey test (T: treatment, S: salinity, C: control).

After 14 days of exposure to salt stress, the MDA content was observed to have significantly increased in the ungrafted plants (*P< 0.05*). The highest MDA content was determined in the ungrafted SCP-2 genotype following salt stress (29.9 µmol g^{-1} FW). The lowest MDA concentration under salt stress was observed in the SCP-1/TLR-2 combination (13.9 µmol g^{-1} FW).

When the MDA content was considered, the ratio of MDA increased by 834 and 988% when compared to controls in the ungrafted sensitive genotypes, while this rate varied between 330 and 650% when grafted with the tolerant genotypes, and a decrease of 17 and 44% occurred when grafted with the tolerant genotypes (Figure 2). In the control groups, no statistically significant differences were determined in the photosynthetic pigments, such as the Chla, Chlb, and total carotenoid contents, between the plant combinations (Figure 2). However, these parameters significantly decreased with the application of 200 mM NaCl in both the grafted and ungrafted plants. The photosynthetic pigments decreased by 44% and 74%, 50% and 53%, and 55% and 64% in the ungrafted genotypes under salt stress when compared with the control groups. On the other hand, the Chla, Chlb, and total carotenoid contents were less affected by salt stress in the SCP-1 and SCP-2 that had been grafted with rootstock (decrease of 12% and 39%).

In response to salt stress, the antioxidant enzyme activities (SOD, CAT, GR, and APX) increased in both the grafted and ungrafted plants. Moreover, the grafted plants exhibited much higher antioxidant enzyme activities than the ungrafted plants under salt stress (Figure 3). The SOD activity was observed to have increased significantly in the grafted plants, by about 35% and 115%, when compared with the ungrafted salt-stressed plants. In addition to this, the TLR-2 rootstock was noted as a rootstock that exhibited the highest SOD enzyme activity in the SCP-1 and SCP-2 plants (increase of 95% and 115%, respectively). Salt stress resulted in enhanced CAT, GR, and APX activities in all of the combinations and the greatest activities were seen in the SCP-2/TLR-2 plants, with differences that were significant when compared to the ungrafted plants and the other graft combinations.

The graft combination, the correlation between shoot and root dry weight, shoot and root Na and Cl ion contents, MDA and the other parameters of melon plants under salt stress conditions are shown in Table 3. Shoot and root dry weight of salt stressed plants were significantly negatively correlated with shoot and root Na and Cl ion contents and MDA concentrations. Similar negative correlations between MDA and all of the other parameters were recorded with the exception of the shoot and root Na and Cl ion contents. On the other hand, all physiological (leaf area, RWC, leaf water potential, Chla, Chlb, and total carotenoid contents, ion content (shoot and root K, Ca, Fe, Cu, Mn, Zn) and antioxidant enzyme activities (SOD, CAT, GR, and APX)

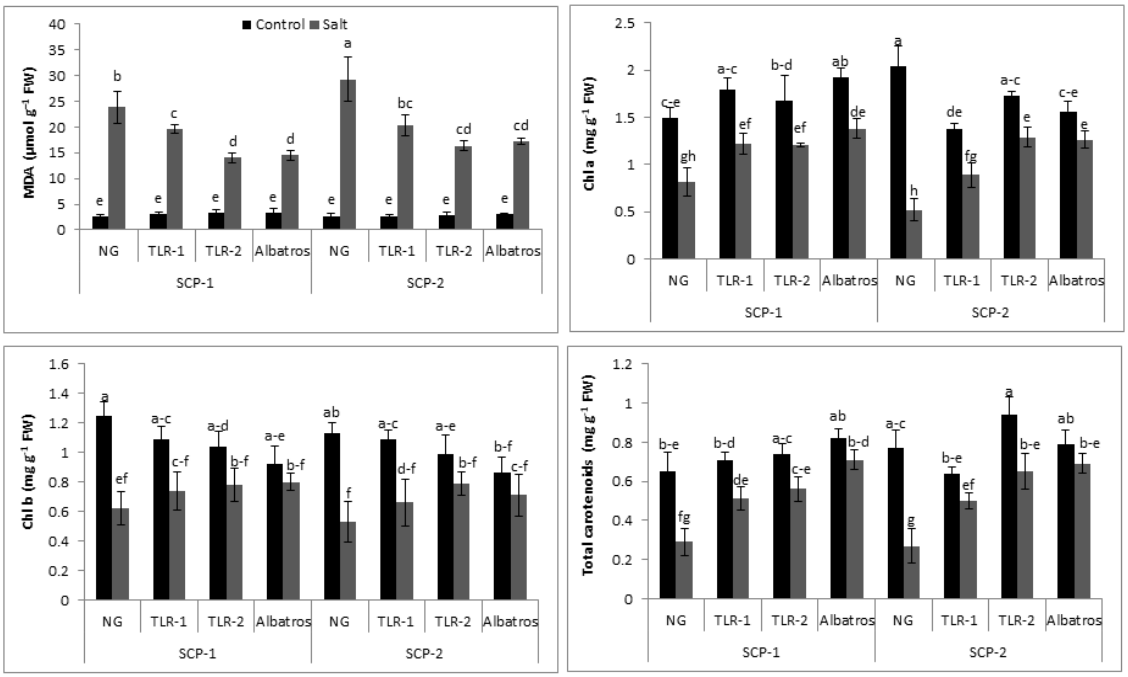

Figure 2. Effects of rootstock on the MDA, Chla, Chlb, and total carotenoid contents of the melon seedlings under salt stress. Each value represents the mean of four replicates. For each parameter, different letters represent statistically significant differences at *P < 0.05* according to the Tukey test.

parameters were significantly positively correlated with shoot and root dry weight under salt stress.

Discussion

Plants are affected by a high level of salinity in two specific ways: 1) high concentrations of salt in the soil interfere with water absorption in the roots, and 2) high concentrations of salt within the plant can lead to the disruption of numerous biochemical and physiological processes, including the absorption and assimilation of nutrients (Carillo et al., 2011). Grafting was observed to have reduced the negative effects caused by the salt stress in this experiment. Under the effects of salt stress, the grafted plants that were tolerant to salinity were observed to have fresh and dry weights, leaf areas, and shoot lengths and diameters that were closer to those of the control plants when compared to the ungrafted plants. The roots of a plant play a vital role in its development, growth, and survival by transporting water and nutrients up to the stem. It can be concluded that the use of rootstocks that are tolerant to salinity can be beneficial under the influence of salt stress. It is a usual condition that the leaf area of plants decreases when they are exposed to salt stress, which could be a mechanism that is used to reduce the amount of water a plant loses during transpiration. This response of the plants can pave the way for toxic ion retention in the roots, which means that the accumulation of such toxic ions is limited in the aerial parts of the plant. Changes in the characteristics of the cell walls and leaf turgor, and a decrease in photosynthesis rates can be given as examples of different responses under the influence of salt stress, all of which induce a decrease in the total area of the leaf (Acosta-Motos et al., 2017). According to the results of this study, when compared to the control plants, it was observed that the leaf area was considerably reduced under the influence of salt stress. This effect was more influential in the ungrafted plants when compared to the grafted plants. It was reported by Böhm et al. (2017) that the highest tolerance against salt stress was seen in grafted plants with watermelon, with an improvement observed in the plant biomass and leaf area, not a reduction, due to salinity. Colla et al. (2010) observed increased dry weight values and a greater number of leaves in all of the grafted watermelon plants in their study when compared to the ungrafted control group, and it was stated by Penella et al. (2016) that a reduction in the leaf area of ungrafted plants was observed when they were grown under stress, and this condition did not change in any of the combinations of grafted pepper plants. Hence, grafting can reduce the effects of stress on melon growth by salinity. The results showed that grafting the plants onto salt-tolerant TLR-1 and TLR-2 was a successful strategy to improve the tolerance of the salt-sensitive melon seedlings (Table 1). Moreover, grafting plants on to the salt tolerant varieties was an effective approach to increase the salt tolerance of the melon seedlings, which was also found to have improved salt tolerance in tomato, eggplant, and cantaloupe (Zhu et al., 2008).

One of the easiest agricultural parameters used to screen plants for salt or drought tolerance is the RWC (Nxele et al., 2017). When transpiration surpasses water absorption, a decrease in cell turgor occurs resulting in lower levels of RWC and cell volume. Low RWC and turgor causes a delay in plant growth and thus, stomatal conductance also decreases (Hammad and Ali, 2014).

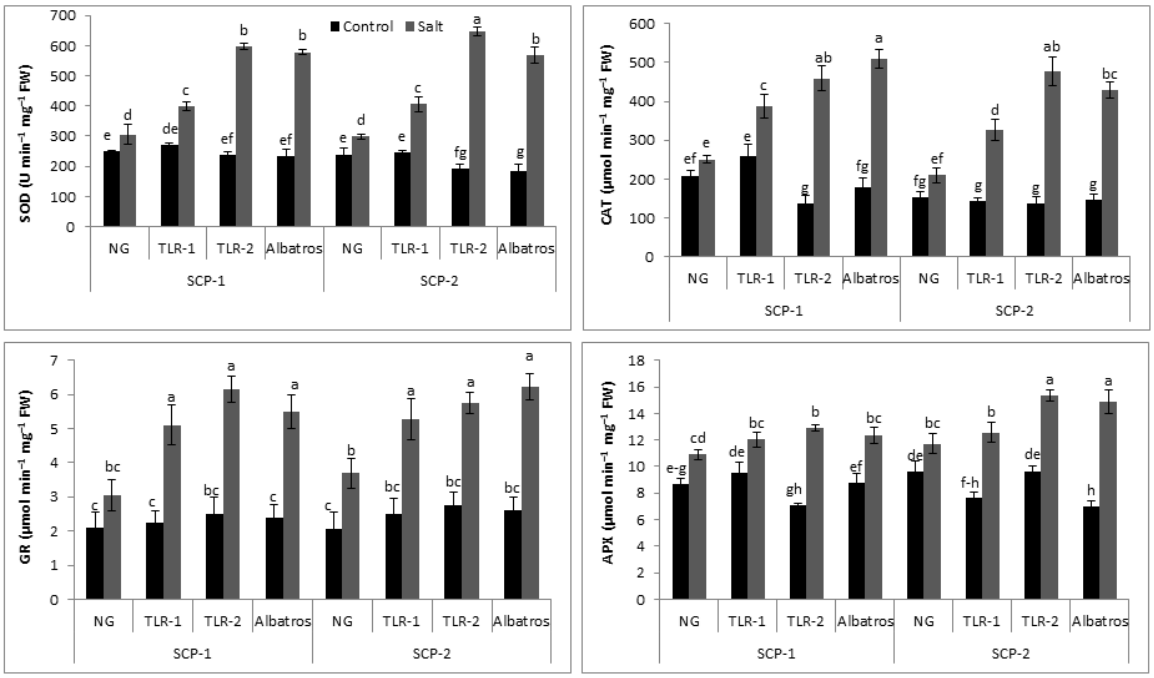

Figure 3. Effects of rootstock on the SOD, CAT, GR, and APX enzyme activities of the melon seedlings under salt stress. Each value represents the mean of four replicates. For each parameter, different letters represent statistically significant differences at *P <0.05* according to the Tukey test.

Santa-Cruz et al. (2002) recorded a 35% improvement in the leaf water content of salt-stressed grafted plants while the scion possessed a salt-incorporating character comparable to that of self-grafted plants. Regulation of the plant Ψw is vital, not simply due to its role in the determination of the response that the plant has salinity, drought, and other stressors, but the effect it has on metabolic processes, which include plant growth as the result of turgor-driven cell expansion (Martinez-Vilalta and Garcia-Forner, 2016). Plants that are under the influence of salinity and water deficit demonstrate mild dehydration signified by lower Ψw, because water uptake is more difficult, and the solution of the soil contains less available water (Acosta-Motos et al., 2017). The exposure of plants to water stress and/or salt stress resulted in the greatest negative values for the leaves and stems of the plants, because both passive hydration and salt gathering caused a reduction in the Ψw of the leaves. To adjust the osmotic potential, the inorganic ion uptake encountered a lower cost of energy than that matched through the synthesis of organic molecules in the cells (Penella et al., 2015). It was stated by Penella and Calatayud (2018) that the salt tolerance of the scions was affected by the features of the rootstock because stomatal functions were controlled effectively; hence, contributing to a change in the hormonal signaling of plants from the roots to the shoots. The grafted plants of cucumber were observed to have higher rates of photosynthesis and stomatal conductance under the influence of salt stress in comparison with the ungrafted plants (Yang et al., 2006). According to previous studies, it was observed that both stomatal conductivity and values of leaf Ψw were reduced; however, the reduction was slightly less in the grafted plants under drought stress (Kiran et al., 2017).

The capability that a plant has to grow under conditions such as high salt stress is related to the ability that the plant has to reserve, categorize, expel, and mobilize Na ions (Zhu et al., 2008). An efficient way to boost the tolerance of plants to salt is grafting onto tolerant rootstock. In previous studies, the latent mechanisms responsible for the advantageous effects of grafting were examined by researchers from the point of view of the changed homeostasis of ions in the roots when compared to the scion, because the roots of the plants have a vital role in regulating the gathering of Na in the shoots (Niu et al., 2017). In the current study, the highest accumulation of Na in the roots and leaves was determined in the ungrafted plants (Figure 1). Hence, the roots of the grafted melon seedlings played a significant role in reducing Na transport to the roots and leaves, which meant that they were highly important for mitigating Na toxicity. The lowest accumulation of Cl^- was seen in the grafted plants, whereas the highest accumulation of Cl^- was observed in the ungrafted plants. In addition, it was stated that the plants managed to prevent Cl^- accumulation in the tissues of their leaves and roots after the process of grafting onto the rootstocks. It was reported by Penella & Calatayud (2018) that the reduction in the growth of grafted pepper plants under salt stress was mainly associated with the low uptake of salt ions when compared to the ungrafted plants. It was reported by Edelstein et al. (2011) that the most frequent grafting application used for vegetables to improve tolerance to salt was on tomato plants. K, which is a vital macronutrient, importantly partakes in stomatal behaviors, osmoregulation, enzyme activities, and the cells

Table 3. The graft combination, the correlation between shoot and root dry weight, shoot and root Cl and Na ions contents, MDA and other parameters of melon plants under salt stress condition

	Shoot Dry Weight	Root Dry Weight	Shoot Cl	Shoot Na	Root Cl	Root Na	MDA
Shoot Fresh Weight	0.946$^{***\&}$	0.663***	-0.763***	-0.761***	-0.687***	-0.613**	-0.770***
Shoot Dry Weight	1.000	0.652***	-0.781***	-0.800***	-0.690***	-0.618**	-0.832***
Root Fresh Weight	0.803***	0.899***	-0.603**	-0.570**	-0.459**	-0.552**	-0.562**
Root Dry Weight	0.652***	1.000	-0.338^{ns}	-0.283^{ns}	-0.193^{ns}	-0.342^{ns}	-0.298^{ns}
Leaf Area	0.895***	0.579**	-0.830***	-0.807***	-0.693***	-0.762***	-0.778***
RWC	0.833***	0.418*	-0.878***	-0.839***	-0.672***	-0.592**	-0.841***
Leaf Water Potential	0.872***	0.347^{ns}	-0.852***	-0.866***	-0.722***	-0.570**	-0.878***
Shoot Cl	-0.781***	-0.338^{ns}	1.000	0.893***	0.792***	0.689***	0.835***
Shoot Na	-0.800***	-0.283^{ns}	0.893***	1.000	0.756***	0.720***	0.901***
Shoot K	0.858***	0.480*	-0.825***	-0.789***	-0.654***	-0.628**	-0.742***
Shoot Ca	0.820***	0.621***	-0.795***	-0.788***	-0.748***	-0.696***	-0.744***
Shoot Fe	0.538**	0.250^{ns}	-0.578**	-0.710***	-0.563**	-0.700***	-0.649***
Shoot Cu	0.231^{ns}	0.332^{ns}	0.161^{ns}	0.044^{ns}	0.032^{ns}	0.229^{ns}	-0.041^{ns}
Shoot Mn	0.661***	0.304^{ns}	-0.753***	-0.684***	-0.513*	-0.403*	-0.673***
Shoot Zn	0.860***	0.530**	-0.734***	-0.785***	-0.646***	-0.661***	-0.731***
Root Cl	-0.690***	-0.193^{ns}	0.792***	0.756***	1.000	0.642***	0.778***
Root Na	-0.618**	-0.342^{ns}	0.689***	0.720***	0.642***	1.000	0.634***
Root K	0.787***	0.437*	-0.877***	-0.845***	-0.735***	-0.639***	-0.778***
Root Ca	0.632***	0.317^{ns}	-0.554**	-0.754***	-0.630***	-0.685***	-0.667***
Root Fe	0.856***	0.696***	-0.620**	-0.644***	-0.520**	-0.497**	-0.659***
Root Cu	0.857***	0.596**	-0.768***	-0.807***	-0.609**	-0.594**	-0.765***
Root Mn	0.813***	0.595**	-0.770***	-0.754***	-0.604**	-0.522*	-0.736***
Root Zn	0.740***	0.842***	-0.568**	-0.511**	-0.402*	-0.379*	-0.498**
MDA	-0.832***	-0.298^{ns}	0.835***	0.901***	0.778***	0.634***	1.000
Chla	0.753***	0.298^{ns}	-0.833***	-0.859***	-0.701***	-0.624***	-0.860***
Chlb	0.459**	0.098^{ns}	-0.565**	-0.552**	-0.657***	-0.316^{ns}	-0.523**
Carot	0.835***	0.535**	-0.867***	-0.781***	-0.592**	-0.635***	-0.779***
SOD	0.909***	0.494**	-0.798***	-0.816***	-0.748***	-0.671***	-0.828***
CAT	0.847***	0.315^{ns}	-0.870***	-0.897***	-0.757***	-0.639***	-0.873***
GR	0.823***	0.551*	-0.760***	-0.756***	-0.513**	-0.684***	-0.725***
APX	0.704***	0.759***	-0.521**	-0.423**	-0.483**	-0.512**	-0.442*

$^{\&}$Levels of significance are represented by *P< 0.05* (*), *P< 0.01* (**), and *P< 0.001* (***) with n.s. meaning not significant.

expansion (Huang et al., 2016). In the current study, in both the grafted and ungrafted plants, the leaf K content decreased with an increase in the NaCl concentration, and the leaf K content was higher in the grafted plants when compared to the ungrafted plants under salt stress (Figure 1), which showed that grafting promoted K transportation to the leaves and reduced K deficiency. Calcium plays a vital role in the formation of cell walls and relations among cells, while it also acts as a regulator in the cation-anion balance and as a catalyst for some enzymes. The decline in the contents of Ca in all of the combinations was assumed to have been a result of the transpiration rate decline under circumstances such as salt stress (Maggio et al., 2007). Under salt stress, some rootstocks have shown an ability to regulate Na partitioning from the shoots to younger and older leaves, and to achieve higher Ca to Na and K to Na ratios in the tips, fruit, and younger leaves when compared to ungrafted leaves. Micronutrients are vital for the growth of plants and they play roles in almost all metabolic and cellular processes, including energy metabolism, both primary and secondary metabolism, cellular defense, regulation of genes, processing of hormones, and transduction and reproduction of signals, in addition to being a catalyst in many antioxidant enzymes (Shahverd et al., 2020). With regards to the micronutrient concentrations in leaves and roots, the data herein showed a general increase under salt stress, except in the Cu contents of the leaves, which decreased in the grafted plants (Table 1). Several studies in the literature have reported that specific grafting combinations were more significantly efficient with regards to the absorption and transportation of nutrients like N, K, Ca, Fe, and other micronutrients, to the shoots when compared with ungrafted plants (Al-Juthery et al., 2019).

Growth inhibition in plants is the result of decreased chlorophyll content, possibly due to ROS-induced chlorosis, photo-reduction, and triplet chlorophyll formation, which cause serious damage to photosystems I and II, and the formation of chlorophyll in plants (Singh et al., 2018). In the decreased chlorophyll contents were observed in the grafted and ungrafted plants under salt stress, while in the grafted salt-tolerant plants, and more specifically in SCP-2/TLR-2, a high chlorophyll content was determined (Figure 2). These results indicated that grafting mitigated the inhibition of photosynthesis that occurred due to salt stress. Carotenoids essentially play a critical part in light harvesting and oxidative damage protection through the deactivation of $^1O_2$, satisfying the excited triplet state in chlorophyll, and the enhancement of carotenoid synthesis as a way of protecting itself from photo-damage caused by cell division arrest when exposed to salt stress (Singh et al., 2018). Ulas et al. (2020) indicated that higher chlorophyll (7.4%) and carotenoid contents (9.4%) of the grafted melons as compared with the nongrafted ones under salt stress.

A biomarker used for oxidative stress-induced lipid peroxidation is MDA content measurement or quantification (Kaushal, 2019). During salt stress, the main targets of ROS are membrane phospholipids that contain polyunsaturated fatty acids. The results of this include the degradation of fatty acids and lipid

peroxidation, which generate a number of cytotoxic products like MDA. In the current study, the lipid peroxidation of the melon plants increased under salt stress. It was noted, however, that the grafted plants exhibited decreased MDA contents under salt stress when compared to the ungrafted salt-stressed plants, which suggested lower ROS accumulation and less damage to the membranes of the grafted plants (Figure 2). This increased effect on the accumulation of MDA could have been related to the higher accumulation of secondary metabolites, resulting in better stabilized subcellular structures, such as membranes and proteins, scavenge-free radicals, and cellular redox buffering potential under salt stress (Islam et al., 2016). Reducing the MDA content in grafted, stressed plants can be an effective mechanism for mitigating the activation of plant defenses. This process also guarantees membrane integrity and reduces the leakage of essential ions (Penella et al., 2016; Zhang et al., 2019). Similar results were also demonstrated by Ulas et al. (2020), who elucidated that our results clearly indicate that grafting with the *Cucurbita maxima × C. moschata* rootstocks had pronounced contributions to the biochemical responses of the scions (melon) under both control and salt stress conditions. ROS are produced as a result of the oxidative stress caused by, in this study, salt. High concentrations of ROS damage DNA, proteins, carbohydrates, and lipids, and hence their concentrations in plant cells must be controlled. ROS elimination, damage management, and repair can be controlled by detoxification signaling (Yang & Guo, 2018). In melon tests, biochemical properties, such as chlorophyll, MDA content, and antioxidant enzyme activities, such as CAT, SOD, and APX, were shown to increase under salt stress when compared with self-grafted and ungrafted plants (Fu et al., 2018). The SOD, CAT, GR, and APX activities of the grafted plants, SCP-1/TLR-2 and SCP-2/TLR-2, were much higher in the current sample than those of the other combinations under salt stress. As a result of the increased antioxidant system activity in grafted plants, the MDA concentration was significantly decreased when compared to the ungrafted plants (Figure 3), indicating less oxidative harm. This suggested that the antioxidant enzyme mechanism worked efficiently in the grafted plants to scavenge overproduced H_2O_2, and thus protected the plants against toxicity to ROS under salt stress. The salt-tolerant grafted plants acquired improved salt stress tolerance as a result of their improved antioxidant system, which led to improved plant growth. According to He et al. (2009), grafted tomato plants were less affected by salt stress and rootstock efficiency in alleviating oxidative stress damage was the result of increased CAT activity and ascorbate-glutathione cycle-involved enzymes, like APX and GR. The ability of grafted plants to alleviate the inhibition effects of salinity can be related to more efficient photosynthesis and enhanced enzymatic antioxidants in grafted plants (Shu et al., 2016). Yanyan et al. (2018) reported that the enhancement of salt tolerance in rootstocks was connected to antioxidant system activation, including those that scavenged ROS, such as POD, SOD, APX, and CAT. This suggested that the SOD/CAT detoxification mechanism worked efficiently in the salt-stressed plants that had been grafted with rootstock for the detoxification of high H_2O_2 levels, and therefore protected the plants from ROS toxicity. The grafted plants seemed to have achieved improved salt stress tolerance via the development of a strengthened antioxidant system, which then resulted in better development.

Conclusion

In the current study, significant differences were observed in the physiological and biochemical reactions in the grafted plant combinations under salt stress. The salt tolerance increase in the grafted plants was the result of using salt-tolerant rootstock, because the scions that were grafted onto the different rootstocks responded somewhat differently to salinity. Grafting can mitigate ion toxicity via the limitation of Na transport and, in some instances, Cl transport, to the shoots, and store the Na and Cl in the roots, which is a common mechanism of tissue tolerance. An effective antioxidant system that can reduce oxidative damage is significant for improving tolerance to salt in grafted plants. The results of the current study have demonstrated that grafting onto salt-tolerant rootstocks could be a technique that is an efficient, effective, and environmentally friendly way to eliminate or reduce yield losses as a result of salinity.

Author Contribution

Conceptualization: ŞK, ŞŞE, Investigation: ŞK, EK, Formal Analysis: ŞK, EK, Resources: ŞK, EK, Writing – Original Draft Preparation: ŞK, Writing – Review & Editing: ŞŞE. All authors read and approved the final manuscript.

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RESEARCH PAPER

Potential biomarkers for lung adenocarcinoma identified by integrative transcriptomics analysis

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Abstract

Lung cancer is one of the most occurring and death-causing cancers worldwide. Despite the progress, survival rate is still low due to the late diagnosis. The aim of this study is to develop a computational framework to identify potential prognostic biomarkers for lung adenocarcinoma (LUAD). Gene expression profiles obtained from three independent studies were analyzed to find differentially expressed genes (DEGs) in LUAD. Disease-specific protein-protein interaction (PPI) network was constructed among common DEGs and hub proteins were identified. Gene expression data was integrated with the human transcriptional regulatory network (TRN) to identify key regulatory elements and construct disease-specific TRN. Hub proteins that were also present in TRN of LUAD were considered as potential biomarkers and assessed by survival analysis. *AURKA, CAV1, CLU, ENO1, FHL1, FHL2, LMO2, MYH11, NME1* and *SFN* were discovered as biomarkers for LUAD, and survival analysis not only indicated their significant prognostic performance as a group, but also revealed their contribution to the discrimination of risk groups. Our findings suggested that identified biomarkers could be valuable in LUAD progression and they should be considered for further experimentation.

Introduction

Lung cancer is one of the most malignant tumors and the leading cause of cancer-associated deaths (Yan et al., 2019). Lung cancer is mainly classified as small cell and non-small cell lung cancer (NSCLC). NSCLC, which accounts for approximately 85% of all the patients, has three main pathological subtypes, including adenocarcinoma (AD), squamous cell carcinoma (SCC), and large cell carcinoma (LCC). When compared to others, AD is the most common type (approximately 50%) (Cagle et al., 2013). Despite the increased understanding of the molecular mechanisms associated with lung cancer and the improvements in traditional treatment, the overall survival rate still remains low. Most patients are diagnosed in a late stage. Therefore, the early detection and diagnosis of the disease can improve the prognosis, increase the survival rate and lower the mortality rate (Villalobos & Wistuba, 2017; Yan et al., 2019).

Biological cancer markers are used for diagnostic, prognostic and treatment purposes (Villalobos & Wistuba, 2017). The targeted therapies in patients with *EGFR* mutations and/or *ALK* translocations improved survival (Patel et al., 2015), suggesting that the genomic biomarkers can serve not only for early diagnosis of the disease, but also for targeted therapies (Li et al., 2019; Villalobos & Wistuba, 2017). Moreover, the identification of the mutations, gene amplifications, deletions, or the presence of the fusion genes, which are accepted as the genetic risk factors associated with lung cancer would lead to developing treatments (Otálora-Otálora et al., 2019). Therefore, the search for novel biomarkers is of great significance and new methods to identify biomarkers with high prognostic performance need to be developed.

Microarray technology has been extensively used to identify genes with altered expressions during tumorigenesis, and therefore, enhances our understanding of the genetics and the molecular biology of cancer (Cieślik & Chinnaiyan, 2018). The identification of the transcriptionally dysregulated genes and their associated biological processes and signaling pathways allow us to understand the expression patterns related to tumor's biological state and the patients' survival (Otálora-Otálora et al., 2019). Moreover, the integration of transcriptome with biological and regulatory networks provide an opportunity to discover novel prognostic biomarkers and therapeutic strategies (Gov et al., 2017).

In the present study, a computational framework was developed to identify potential biomarkers for LUAD (Figure 1). For this purpose, firstly, gene expression data from three independent studies were analyzed and common DEGs, biological processes and molecular pathways involved in LUAD were determined. Secondly, a disease-specific PPI network was reconstructed with common DEGs and hub proteins were determined via simultaneous analysis of twelve node scoring metrics. Then, key regulatory elements involved in LUAD were identified by statistical analysis using hypergeometric probability distribution function and disease-specific TRN was constructed by common key regulatory elements interacted with common DEG targets. A total of ten genes that were identified as hub genes of LUAD specific PPI network and also present in LUAD specific TRN, were determined as potential biomarkers. Finally, survival analysis was conducted to evaluate their prognostic capabilities and biomarker genes showed significant prognostic performances and high capabilities in classifying risk groups.

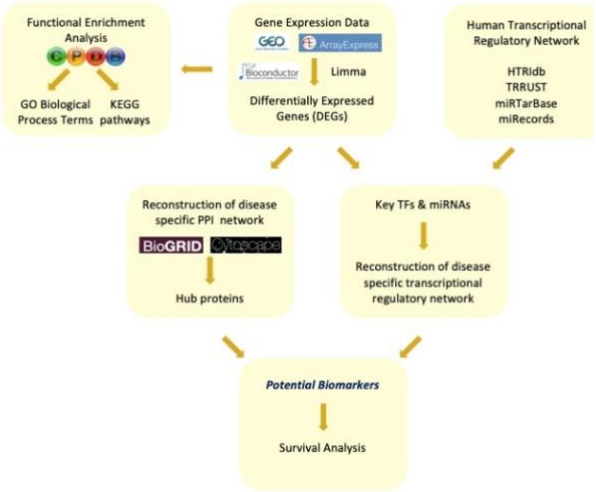

Figure 1. Schematic illustration of the system-based integrative analysis carried out in the present study.

Materials and Methods

Gene expression data collection and analysis

Gene expression datasets from three independent studies [GSE118370 (Xu Liyun et al., 2018), E-MTAB-5231 (Willuda et al., 2017), and GSE40791 (Y. Zhang et al., 2012)] associated with LUAD were selected from ArrayExpress or Gene Expression Omnibus databases. The datasets obtained by Affymetrix Human Genome U133 Plus 2.0 arrays were used to avoid altered gene expressions due to microarray differences. GSE118370 was consisted of tumor and paired adjacent non-tumor tissues from 6 LUAD patients. E-MTAB-5231 contained 22 NSCLC samples, including AD and SCC samples, and 18 normal lung tissues adjacent to tumor samples. Since AD subtype was specifically investigated in this study, SCC samples were excluded from this dataset and data obtained from samples of two stage I and seven stage II LUAD patients were analyzed. GSE40791 contained 94 tumor tissues from 69, 12, and 13 stage I, II, and III LUAD patients, respectively and 100 non-tumor tissues. Two independent datasets, GSE63459 (Robles et al., 2016) containing 33 stage I LUAD tissues and their non-tumor adjacent tissues, and GSE75037 (Girard et al., 2017) containing 83 samples from LUAD tissues and their non-malignant adjacent tissues were used to assess the expression of candidate biomarker genes. By using these two datasets, principal component analysis (PCA) was performed via R software (*R Core Team*, 2020) to elucidate whether the candidate biomarker genes could discriminate LUAD tissues and non-tumoral tissues according to gene expression levels.

Each gene expression dataset was normalized independently and the associated significantly expressed genes were determined using R/Bioconductor (Gentleman et al., 2004). Quantile normalization was performed by RMA (Bolstad et al., 2003) option of the *affy* package (Gautier et al., 2004) and multiple testing option of LIMMA (Smyth, 2004) was used for statistical analysis. The false-discovery rate was controlled by Benjamini-Hochberg's method (Benjamini & Hochberg, 1995). An adjusted *P-value* threshold of 0.05 was used to determine the significance of gene expression and the genes showing at least 2-fold change (FC) in their expression levels were identified. The genes satisfying both *P-value* and FC thresholds, were defined as DEGs. Scatter volcano plots were constructed via R using log2 FCs and corresponding *P-values* of all genes in gene expression data. A heat map that represents gene expression profiles of common DEGs among all samples, was plotted using R. Overrepresentation analyses were performed by ConsensusPathDB (Kamburov et al., 2013), and a *P-value* threshold of 0.05 was used to identify significantly enriched KEGG pathways and GO biological processes associated with DEGs.

Reconstruction of disease-specific protein-protein interaction network

To construct a PPI network of LUAD, physical interactions between the proteins encoded by common DEGs were extracted from BioGrid (Stark et al., 2006) database release 4.2.191. This network was further analyzed and visualized via Cytoscape (Shannon et al., 2003) version 3.7.2. To identify hub proteins, node

scores were determined using CytoHubba (Chin et al., 2014). All nodes were ranked based on twelve scoring metrics, including degree, maximal clique centrality (MCC), maximum neighborhood component (MNC), density of maximum neighborhood component (DMNC), betweenness, edge percolated component (EPC), bottleneck, eccentricity, closeness, radiality, clustering coefficient (CC) and stress. Top ten proteins of each scoring metric were isolated and the proteins that were commonly identified by at least five scoring methods were determined as hub proteins.

Reconstruction of disease-specific transcriptional regulatory network

The reported transcription factor (TF)-target gene interactions in HTRIdb (Bovolenta et al., 2012) and TRRUST v2 (Han et al., 2018) databases, as well as microRNA (miRNA)-target gene interactions with strong experimental evidences deposited in miRTarBase release 8.0 (Chou et al., 2018) and miRecords (Xiao et al., 2009) databases were used to reconstruct human TRN. The constructed network was composed of 25669 interactions between 827 TFs and 12659 genes; and 9905 interactions between 844 miRNAs and 3269 genes. For each gene expression dataset, TF-target DEG and miRNA-target DEG interactions were extracted from human TRN. Enrichment analysis were conducted using hypergeometric distribution function, and *P-value* threshold of 0.05 was used to identify key regulatory elements, i.e., TFs and miRNAs, in the presence of LUAD. Disease-specific TRN was constructed by employing key regulatory elements that were found to be common in three datasets interacted with common DEGs.

Identification of potential biomarkers for LUAD

Candidate biomarkers for LUAD were determined by simultaneous analysis of disease-specific networks, i.e., PPI and TRN. Genes that encode hub proteins and are also present in the disease-specific TRN were considered as potential biomarkers for LUAD. The disease involvements of key TFs and biomarker genes as well as their prognostic capabilities in cancer were investigated via Human Protein Atlas available from http://www.proteinatlas.org (Uhlen et al., 2017). Human miRNA and disease associations were identified by the Human microRNA Disease Database (HMDD v 3.2) (Lu et al., 2008).

Survival analysis

The prognostic capabilities of potential biomarkers were assessed using gene expression data containing 475 samples obtained from TCGA. Cox proportional hazards regression analysis was carried out via SurvExpress (Aguirre-Gamboa et al., 2013), and according to the prognostic index, patients were classified as high- and low-risk groups. Gene expression levels of risk groups and survival times were visualized by box-plots and Kaplan-Meier plots, respectively. A log-rank *P-value* cut-off was maintained as 0.05 to define the statistical significance of survival.

Results

Analysis of significantly and differentially expressed genes

The gene expression profiles of tumoral and non-tumoral lung tissues obtained from three independent studies were comparatively analyzed. A total of 1349 genes (425 up, 924 down), 1447 genes (666 up, 781 down), 2202 genes (777 up, 1425 down) were identified as DEGs in GSE118370, E-MTAB-5231, GSE40791, respectively, and scatter volcano plots were plotted to illustrate the distribution of each gene according to the log2FC and –log (*P-value*) values (Figure 2a). Although the numbers of DEGs among datasets were incompatible, 432 DEGs (83 up- and 349 down-regulated) were found to be significantly expressed in common (Figure 2b). A heat map showed the expression profiles of common 432 DEGs identified in the analysis. The common 432 DEGs, including 349 significantly down-regulated genes and 83 significantly up-regulated genes, could effectively distinguish LUAD samples from normal samples (Figure 2c). Since common DEGs might hold important information on LUAD, the significantly enriched GO biological process terms and KEGG pathways (Figure 3a) and protein classes (Figure 3b) associated with these genes were identified.

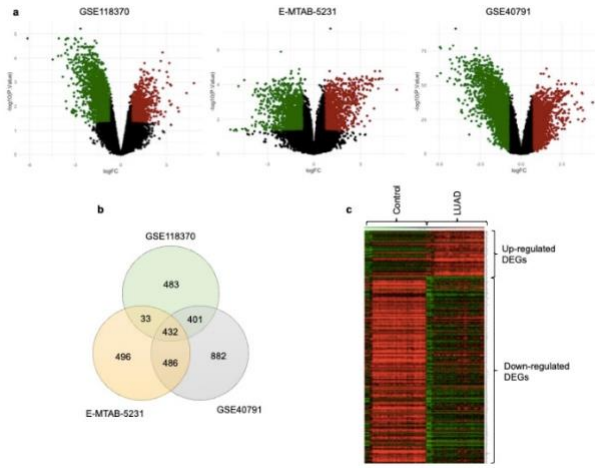

Figure 2. a) The volcano plots for the transcriptome datasets. The x-axis represents the log2 transform of fold change ratios; the y-axis represents the log10 transformed adjusted *P-value*. The red and green colored dots represent the up- and down-regulated DEGs, respectively. **b)** Venn diagram representing the comparison of DEGs in the datasets. **c)** Heatmap representation of common DEGs.

A total of 83 genes, which were commonly induced in LUAD tissues, were found to be significantly associated with cell cycle, DNA damage, cell adhesion, and extracellular matrix (ECM) related processes. The top significant processes related to the commonly repressed 349 genes were angiogenesis, cell adhesion,

vasculogenesis, and ECM organization. Moreover, pathway analysis indicated vascular smooth muscle contraction, ECM-receptor interaction, cGMP-PKG signaling pathways among the significantly enriched KEGG pathways of 432 common DEGs.

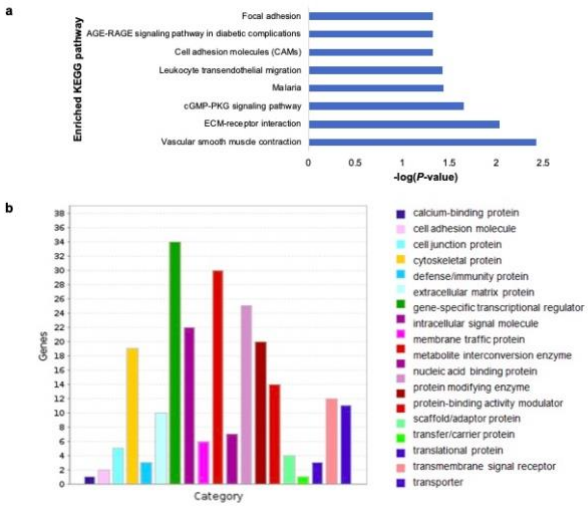

Figure 3. a) Significantly enriched KEGG pathways of common DEGs. **b)** Panther protein classes of common DEGs.

Identification of hub proteins

LUAD specific PPI network constructed by collecting interactions between the proteins encoded by common DEGs, contained 204 interactions between 165 proteins (Figure 4). Hub proteins, which might have important roles in the progression of the disease, were determined by simultaneous investigation of twelve scoring metrics. The proteins that are among the top ten proteins determined by at least five scoring metrics were identified as hub proteins. Aurka, Cav1, Chmp4c, Clu, Eno1, Fhl1, Fhl2, Lmo2, Myh10, Myh11, Nme1 and Sfn were found as hub proteins.

Identification of key regulatory elements

Transcriptional and post-transcriptional regulatory changes in the presence of LUAD were elucidated by integrative analysis of gene expression data and the constructed human TRN. Key regulatory elements for each dataset were determined by extracting TF-DEG and miRNA-DEG interactions from human TRN and calculating hypergeometric probability in the disease state considering a statistical significance threshold of *P-value* < 0.05. Key miRNAs, including miR-103a-3p, miR-135a-5p, miR-200c-3p, miR-203a-3p, miR-204-5p, miR-21, miR-223, miR-25-3p and miR-29a-3p were identified in LUAD (Table 1). Disease associations of key miRNAs were investigated via HMDD and all of them were found to be linked to either lung neoplasms or NSCLC, including LUAD for six of them. Moreover, two of the key miRNAs, miR-200c and miR-21, were previously reported among the significantly expressed miRNAs in stage I LUAD patients and survival analysis indicated that miR-21 was significantly associated with the prognosis of LUAD (Robles et al., 2016). A total of 34 TFs were identified as key regulatory TFs. Top five of the key TFs were also presented in Table 1. The gene products of 25 key TFs were previously determined as prognostic markers of various cancers with Fosl1 being a prognostic marker of lung cancer. Moreover, defects in *IRF1* was associated with lung cancer. TRN of LUAD was constructed by isolating the interactions between key regulatory elements and common DEGs from human TRN. The disease-specific TRN contained 680 interactions between 43 key regulatory elements (34 TFs and nine miRNAs) and 306 common DEGs.

Determination of potential biomarkers

Disease-specific PPI network and TRN were used to identify potential biomarkers of LUAD. Genes that encode hub proteins were regarded as potential

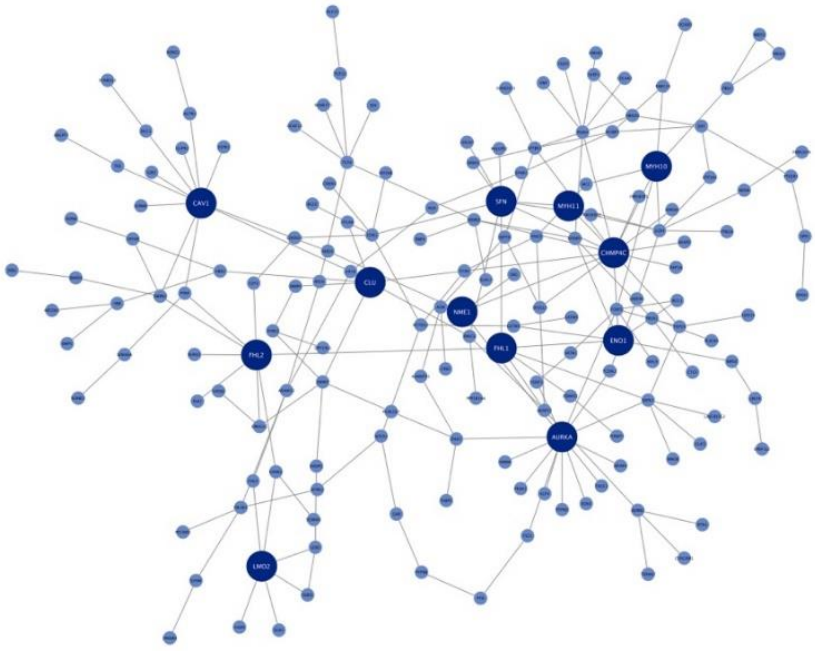

Figure 4. LUAD specific PPI network (dark blue nodes represent hub proteins).

Table 1. Key regulatory elements in LUAD

Key miRNA	Feature
miR-103a-3p	Afflicted with lung neoplasms
miR-135a-5p	Afflicted with lung neoplasms, LUAD
miR-200c-3p	Afflicted with lung neoplasms, lung fibrosis, NSCLC
miR-203a-3p	Afflicted with lung neoplasms, NSCLC, LUAD
miR-204-5p	Afflicted with NSCLC, LUAD
miR-21	Afflicted with lung neoplasms, NSCLC, SCC
miR-223	Afflicted with lung neoplasms, NSCLC, LUAD
miR-25-3p	Afflicted with lung neoplasms, NSCLC, LUAD
miR-29a-3p	Afflicted with lung neoplasms, lung fibrosis, NSCLC, LUAD
Key TFs	**Prognostic marker**
AR (Androgen receptor)	renal cancer (favorable) and liver cancer (favorable)
RELA (RELA proto-oncogene, NF-kB subunit)	renal cancer (unfavorable) and liver cancer (unfavorable)
SP1 (Sp1 transcription factor)	pancreatic cancer (unfavorable)
ESR1 (Estrogen receptor 1)	endometrial cancer (favorable)
YBX1 (Y-box binding protein 1)	renal cancer (unfavorable) and liver cancer (unfavorable)

biomarkers only if they were members of LUAD specific TRN. *AURKA, CAV1, CLU, ENO1, FHL1, FHL2, LMO2, MYH11, NME1* and *SFN* were discovered as biomarkers and their functions, disease involvements and prognostic capabilities in cancer were presented in Table 2.

Biomarker genes were also analyzed for enriched GO biological processes. Cell cycle, DNA damage checkpoint, DNA integrity checkpoint, cell death, apoptotic signaling pathway, cell proliferation, lipid metabolic process, response to lipid, reactive oxygen species biosynthetic process, cardiocyte differentiation, circulatory system development, heart development, and muscle contraction were found among the significantly enriched GO biological processes.

Candidate biomarker genes were also assessed by using two independent datasets, GSE63459 and GSE75037 containing samples from LUAD tissues and their non-tumoral adjacent lung tissues. All biomarker genes were significantly expressed in GSE63459 and the expressions of all biomarker genes, except *ENO1* were found to be significantly altered in GSE75037. The capabilities of biomarker genes to discriminate LUAD and non-tumoral lung tissues were assessed by PCA. PCA was carried out based on the expression profiles of biomarker genes in GSE63459 and GSE7503, and the first three principal components describing at least 74% of the total variance were considered. The biomarker genes as a group showed high potential in discriminating LUAD tissues from non-tumoral adjacent lung tissues (Figure 5).

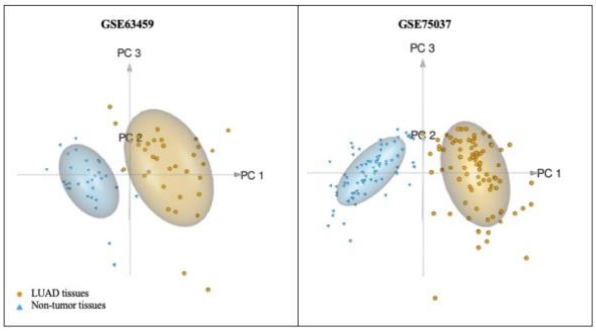

Figure 5. PCA was applied using the gene expression profiles of biomarker genes and the first three principal components could separate LUAD tissues from non-tumoral adjacent lung tissues in GSE63459 and GSE75037. Samples from LUAD tissues, and non-tumoral adjacent lung tissues were presented in yellow and blue colors, respectively. Ellipses indicate 95% confidence interval.

Survival analysis of potential biomarkers

The prognostic performances of potential biomarkers were determined via Cox proportional hazards regression analysis performed in SurvExpress. Patients were classified as high- and low-risk groups according to prognostic index estimated via expression levels of biomarker genes. Box-plots and Kaplan-Meier plots were used to visualize gene expression levels of risk groups and survival probabilities, respectively (Figure 6). The difference in gene expression between risk groups were compared using t-test and the biomarker genes were found to have significantly different (*P-value* < 0.05) expression levels in high- and

Table 2. Summary of identified biomarker genes

Gene	Prognostic marker
AURKA (Aurora Kinase A)	renal cancer (unfavorable), endometrial cancer (unfavorable), liver cancer (unfavorable) and pancreatic cancer (unfavorable)
CAV1 (Caveolin 1)	lung cancer (unfavorable) and renal cancer (unfavorable)
CLU (Clusterin)	thyroid cancer (favorable)
ENO1 (Enolase 1)	liver cancer (unfavorable) and glioma (unfavorable)
FHL1 (Four and a half LIM domains 1)	urothelial cancer (unfavorable)
FHL2 (Four and a half LIM domains 2)	renal cancer (unfavorable) and head and neck cancer (unfavorable)
LMO2 (LIM domain only 2)	liver cancer (favorable)
MYH11 (Myosin heavy chain 11)	liver cancer (favorable)
NME1 (NME/NM23 nucleoside diphosphate kinase 1)	renal cancer (unfavorable) and liver cancer (unfavorable)
SFN (Stratifin)	renal cancer (unfavorable), liver cancer (unfavorable), endometrial cancer (favorable) and pancreatic cancer (unfavorable)

low-risk groups (Figure 6a). Moreover, analysis indicated the significant prognostic performance of biomarker genes as a group (Hazard ratio = 1.72 and log-rank *P* = 0.0005) (Figure 6b). Biomarker genes showed high performance in classifying the patients with long and short survival and approximately 1.72-fold difference in death rate between these groups was observed.

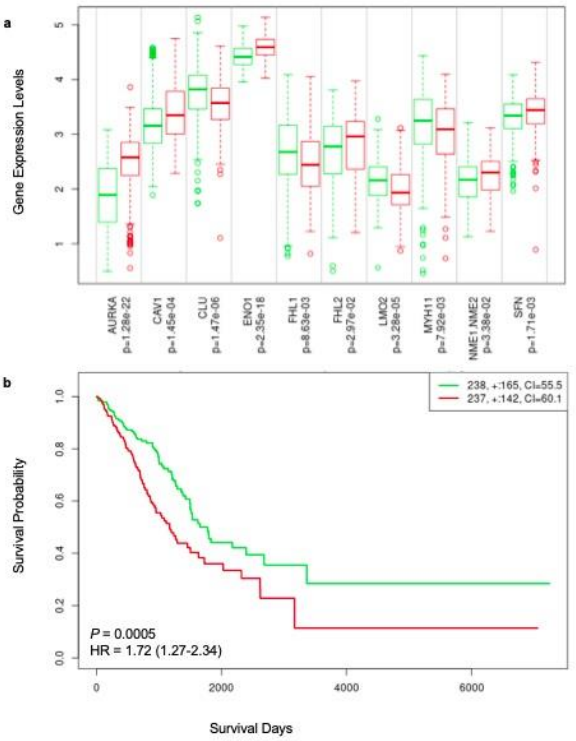

Figure 6. Survival analysis of potential biomarker genes. **a)** Box-plot showing the range and distribution of the gene expression levels of biomarker genes in high- and low-risk groups and *P-value*s representing the differences in biomarker genes' expression levels between high- and low-risk groups. **b)** Kaplan-Meier plot of biomarker genes. Hazard Ratio (HR) was given with a 95% confidence interval. Red and green curves denote high- and low-risk groups, respectively.

Discussion

Lung cancer is the leading cause of cancer associated deaths worldwide (Yan et al., 2019). Due to the high mortality rate, the identification of predictive biomarkers with high prognosis is still an issue. This study developed a computational framework to discover biomarkers for LUAD that is the most common type of lung cancer. The gene expression profiles obtained from healthy and tumoral lung tissues were comparatively analyzed to identify DEGs. Transcriptomics data derived from three independent studies was used to catch diverse processes, pathways, and key molecules that cannot be observed by a single dataset and to reduce the errors associated with a single dataset. Transcriptome, interactome and regulome were used simultaneously for the construction of disease-specific networks, i.e., PPI network and TRN. The integrative analysis of disease-specific networks yielded *AURKA*, *CAV1*, *CLU*, *ENO1*, *FHL1*, *FHL2*, *LMO2*, *MYH11*, *NME1* and *SFN* as potential biomarkers that could be used in the diagnosis and/or prognosis of LUAD and survival analysis revealed their significant prognostic performances.

The analysis of gene expression datasets used in this study revealed 432 common DEGs whose expression patterns were predominantly down-regulated (81%). Functional enrichment analysis of common DEGs revealed three major mechanisms that accompany LUAD: changes in cell adhesion properties, possibility of bone metastasis, and pulmonary vascular remodeling. i) Enrichment analysis indicated cell adhesion, ECM organization and collagen associated processes among the affected processes. Collagen is the most abundant component of ECM, and the modifications in the content and the distribution of collagen due to tumor microenvironment lead to structural changes in ECM during the development of cancer (Lai Xu et al., 2017; S. Xu et al., 2019). Therefore, the induction and repression observed in ECM organization and collagen metabolism were in accordance with the loss of adhesion properties of tumor cells. ii) Bone is one of the most common sites of metastasis and bone metastasis occurs in 30-40% of the advanced lung cancer patients (Macedo et al., 2017). Genes functioning in bone associated processes, such as ossification, fibroblast growth factor, bone remodeling and mineralization were found to be significantly expressed, which might indicate the possible occurrence of bone metastasis in LUAD patients. iii) Significant repression of genes involved in cardiovascular system related processes that might be due to pulmonary vascular remodeling was observed. Pulmonary vascular remodeling frequently accompanies lung cancer and leads to the thickening of blood vessel wall. Since the accumulation of ECM components may cause this type of thickening (Jeffery & Wanstall, 2001; Pullamsetti et al., 2017), the repression of cardiovascular system associated processes together with ECM organization supports the possible changes in the pulmonary vascular structure in LUAD.

Disease-specific networks were constructed by integrating common DEGs with human biological networks. The PPI network was constructed to unveil the interactions among common DEGs and 12 hub proteins were found to be noteworthy in LUAD specific PPI network. Regulatory elements targeting common DEGs were used for the construction of LUAD specific TRN and key regulatory elements, i.e., 34 TFs and nine miRNAs came into prominence in LUAD. Key miRNAs were previously reported to be associated with lung cancer and key TFs were served as prognostic markers in various cancers (Table 1). Therefore, hub genes regulated by key regulatory elements were assessed as candidate biomarkers for LUAD. Since key regulatory elements might also hold considerable information on the molecular mechanisms of the disease, possible regulatory mechanisms were highlighted and the

interactions between key molecules were presented in Figure 7.

When functional enrichment analysis for the discovered biomarkers was conducted, a significant relation between biomarker genes and cell cycle, DNA damage, lipid and cardiovascular system associated processes was observed. The similarity between the processes enriched with biomarkers and that of the common DEGs supported that the discovered biomarkers were representative of LUAD. Therefore, the discovered biomarkers were further investigated to understand their roles in tumor progression. Survival analysis showed that the biomarker genes as a group had high likelihood of being prognostic biomarkers for LUAD. Moreover, the diagnostic capabilities of biomarker genes were evaluated using two independent gene expression datasets and PCA indicated that they could effectively distinguish LUAD samples from normal samples.

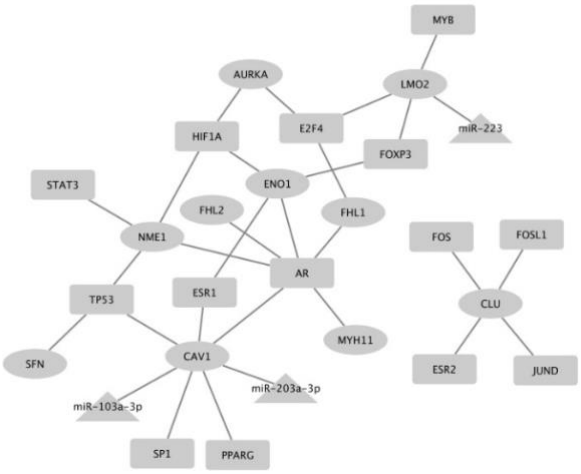

Figure 7. Key regulatory elements targeting biomarker genes. Ellipses, rectangles, and triangles represent biomarker genes, TFs, and miRNAs, respectively.

All discovered biomarkers were reported as prognostic markers of various cancers and among them *CAV1* was classified as a prognostic marker of lung cancer (Table 2). *CAV1* encodes a scaffolding protein that is the main component of the caveolae on the plasma membrane. The protein links integrin subunits to the tyrosine kinase FYN, an initiating step in coupling integrins to the Ras-ERK (extracellular signal-regulated kinases) pathway and promoting cell cycle progression. *CAV1* is associated with migration, invasion, and metastasis in cancers and it functions as a tumor suppressor or promoter depending on the stage of the tumor (Shi et al., 2020). Another metastasis suppressor gene, *NME1*, was also detected as a potential biomarker. *NME1* was previously identified because of its reduced mRNA transcript levels in highly metastatic cells. Although its overexpression was not related to the primary tumor size, the metastatic formation was found to be significantly reduced (Marino et al., 2013).

Aurka and Sfn have several functions during mitosis. *AURKA* encodes a cell cycle regulated kinase that is involved in microtubule formation and/or stabilization at the spindle pole during chromosome segregation. It is involved in tumorigenesis through multiple mechanisms and interactions with various proteins functioning as tumor suppressors and oncogenes (Tang et al., 2017), and it has also been linked to poor differentiation of lung cancer (Lo Iacono et al., 2011). *SFN* encodes a cell cycle checkpoint protein that regulates mitotic translation and plays a role in preventing DNA errors during mitosis in response to DNA damage. The suppression of *SFN* expression resulted in a reduction in cell proliferation in LUAD cell line (Shiba-Ishii et al., 2015).

LMO2 encodes a highly conserved cysteine-rich, two LIM-domain protein that has a central and crucial role in hematopoietic development. All human LIM-domain proteins (Lmo1-4) are associated with progression of various cancers. Although *LMO2* is specifically linked to T cell leukaemia, its interacting partner *GAMA2* was involved in the regulation of tumor development in RAS-mutant NSCLC (Matthews et al., 2013). Fhl1 and Fhl2 are also members of the four and a half LIM only protein family. The involvement of *FHL1* and *FHL2* in cancer development is due to their interactions with cancer-related proteins Smad2-4, which results in enhanced expressions of growth inhibiting genes and a decreased expression of a growth promoting gene *c-myc* (Ding et al., 2009). Previous reports also indicated a significant association between the expression of *FHL2* and the cellular level of p53, which is an important tumor suppressor protein (Cao et al., 2015). The communication among the genes encoding these LIM-domain proteins were regulated by *E2F4* that is a common regulator of *LMO2* and *FHL1* together with *AR* that is a common regulator of *FHL1* and *FHL2* in LUAD specific TRN (Figure 7). Since these biomarker genes and their common regulators might play a notable role in LUAD, we suggested them as novel molecular targets in LUAD. Another hub gene, *ENO1* encodes an alpha-enolase and is also involved in growth control and hypoxia tolerance. *ENO1* also modulates *c-myc* expression and inhibits tumor growth (Zhang L. et al., 2018). The expression of *ENO1* was reported to be significantly higher in lung cancer tissues when compared to benign lung disease tissues, however, its expression was not significantly altered to differentiate the subtypes of lung cancer (Zhang L. et al., 2018).

CLU encodes a secreted chaperone that prevents aggregation of non-native proteins and is involved in several basic biological events such as cell death and tumor progression. Intracellular clusterin also regulates the expression of some genes involved in DNA repair. It was previously associated with lung diseases, including asthma and idiopathic pulmonary fibrosis (Habiel et al., 2017). Moreover, its overexpression was observed in a rat model of pulmonary arterial hypertension and contributed to pulmonary vascular remodeling (Liu et al., 2015).

MYH11 encodes a smooth muscle myosin that functions as a major contractile protein, converting chemical energy into mechanical energy through the hydrolysis of ATP. Myosins have several roles in processes related to tumor invasion, such as cell adhesion and migration. *MYH11* was previously reported to be involved in the contraction of airway smooth muscle in asthma. Moreover, the dysregulation of *MYH11* expression was observed in various cancers with a significant decrease in lung cancer (Nie et al., 2020).

Conclusion

There is an ever-growing interest in the identification of diagnostic and prognostic biomarkers and therapeutic targets for lung cancer. Within the framework of this study, the major molecular mechanisms underlying LUAD were determined via comparative transcriptome profiling, and a computational framework was developed to identify potential biomarkers for LUAD. Disease-specific PPI network and TRN were constructed and integrative analysis of these networks with transcriptome data elicited potential biomarkers. The identified biomarkers in this study did not only represent common DEGs functionally, but also showed significant prognostic performance as a group. Possible regulatory communications that might have vital roles in LUAD were also highlighted. Since the identified biomolecules could be valuable for diagnosis and targeted therapies, they deserve clinical investigation. Further experimentation needs to be carried out to verify biomarkers' diagnostic capabilities and to pinpoint their roles in LUAD progression.

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BIOTECH STUDIES

REVIEW

Genetic resistance of wheat towards plant-parasitic nematodes: current status and future prospects

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Abstract

Plant-parasitic nematodes (PPNs) are one of the major biotic factors that cause significant yield losses in wheat-growing areas worldwide. The major PPN groups causing significant economic losses in wheat quantity and quality are cereal cyst nematodes (CCNs) and root-lesion nematodes (RLNs). Based on their wide distribution, pathogenicity, high occurrence in wheat cultivated areas, they are considered major threats to the global food supply. The economic loss caused by these destructive pathogens ranges from 10 to 100% depending on different agro-ecological conditions such as drought, heat stress, and cold stress. Multidisciplinary management practices are being implemented to manage cereal nematodes (CNs) that range from cultural to molecular strategies. Integration of wheat resistant varieties with appropriate agronomic practices is recognized as the safest and most practical, effective, and applicable management strategy. Nine resistance genes (*Cre1–Cre9*) to CCN are well-documented in the literature. *CreR*, *CreV* and *CreZ* genes are relatively recently characterized from wheat and confer resistance to CCNs. On the other hand, *Rlnn1* is the only resistance gene characterized from wheat that is known to confer resistance towards RLNs. However, breeding for resistance to PPN has numerous challenges that originate from the narrow genetic diversity and difficulty in the process of transferring resistance gene(s) from the source to the target variety. A unique opportunity for wheat genetic improvement was provided due to the availability of genomic resources and the wheat worldwide germplasm collection which includes wild wheat germplasm. Moreover, the presence of several genome-wide association studies and genome editing technologies could also help for further improvement to enhance CNs resistance in wheat. This article provides the latest information regarding the progress made in the identification and characterization of resistance genes from different sources and its utility against both CCNs and RLNs, which will attract the attention of the scientific community and other relevant stakeholders.

Introduction

Wheat is a cereal grain and global staple food. Bread wheat (*Triticum aestivum L.*) is an allohexaploid (~17 Gbp genome size) derived from a combination of three closely related genomes (A, B, and D) formed through multiple hybridizations among these three diverse ancestor species (Feldman et al., 2012). The earliest hybridization happened between *T. urartu* (AA, 2n=14) as the A-genome donor and an unknown species

with the B-genome (BB, 2n=14, presumably *Aegilops speltoides*) (McFadden & Sears, 1946), resulting in the tetraploid ancestor of modern *Triticum* species, wild emmer wheat *T. turgidum* ssp. *Dicoccoides* (AABB, 2n=28), this further hybridized with *A. tauschii* (DD, 2n=14) ending with the modern bread wheat (AABBDD, 2n=42) (Huang et al., 2002; Dubcovsky & Dvorak, 2007; Hussain & Rivandi, 2007; Shewry, 2009; Matsuoka, 2011). Wheat is considered among the most ancient of cultivated cereal crops originating in the Fertile Crescent around 9,600 B.C (Piperno et al., 2004). Based on the FAOSTAT (2019 data, bread wheat is grown on over 215 million hectares of land globally, which is feeding over 40% of the global population (Taheri et al., 2019) contributing approximately to up to 30% of the total world food grain production (FAOSTAT, 2019). It is the main food crop used worldwide and contains the major source of proteins (21%) and calories (19%) in human diets, and provides substantial feed to animals (Shiferaw et al., 2013) compared with other food crops. Due to its high yielding, nutrition value, ease of grain storage, and transformation into wide varieties of food forms, wheat is a vital diet component (Curtis, 2002; Shewry, 2009). Moreover, it provides essential amino acids, minerals, vitamins and dietary fiber (Shewry, 2009; Bockus et al., 2010).

Globally, wheat production needs to be enlarged by 60% to achieve the projected demand by 2050 (Ackerman & Stanton, 2008). World wheat production is boosted mainly due to the wider usage of agricultural technologies and the deployment of improved cultivars and farming practices (Shiferaw et al., 2013). However, wheat production is still limited by both biotic (diseases, nematodes, insect pests, weeds, etc.) and abiotic (adverse climatic, salinity, and edaphic) constraints (Husenov et al., 2020). Among these production limitations, plant-parasitic nematodes (PPNs) were estimated to decrease production by 10% (Whitehead, 1998). Among the PPNs recorded on wheat, CCN and RLN are the major ones, are among the top ten economically important genera (Jones et al., 2013), and were ranked 2nd and 3rd in their economically and scientifically important after the root-knot nematode genera (Dababat & Fourie, 2018). Cereal cyst nematodes (*Heterodera* spp.) (CCNs) have a wide distribution and cause a considerable yield loss in many countries (Hajihassani et al., 2010; Dababat et al., 2015; Pariyar *et al.*, 2016a; Toumi et al., 2017; Renco et al., 2018). The major species of CCN affecting cereal crops including wheat are *Heterodera avenae*, *H. filipjevi* and *H. latipons* (Imren et al., 2013; Toumi et al., 2013; Baklawa et al., 2015; Imren et al., 2019). The major root-lesion nematodes (*Pratylenchus* spp.) (RLN) species are *Pratylenchus thornei*, *P. neglectus*, *P. penetrans*, and *P. crenatus* where all above-mentioned species have a global distribution and sometimes co-exist in a single field (Nicol et al., 2003; Smiley & Nicol, 2009; Dababat et al., 2016). Apart from the CCN and RLN damage on wheat crops individually they are also known to interact with each other as well as with other soil-borne fungal pathogens such as the crown rot fungus (*Fusarium culmorum*), which is known to limit wheat yield in temperate and semi-tropical regions of the world, creating a disease complex (Hajihassani et al., 2013; Dababat et al., 2018).

The efficient management of wheat crop diseases is crucial in maintaining world food supply stability (Ogbonnaya et al., 2008). The wheat crop can be protected from CN damage by various management strategies such as crop rotation, host resistance, cultural practice, biological control, and application of chemicals mainly nematicides (Dababat et al., 2015). The genetic differences in landraces and domesticated wheat cultivars offered resistance against a considerable number of abiotic and biotic stresses (Pariyar et al., 2016b). Integrating resistance genes into breeding resistant lines and wheat cultivars are considered the most fruitful management strategy to lower nematode populations below the economic threshold level (Dababat et al., 2015). Resistance management is an ecologically and friendly approach and achievable through the international active partnership of research groups (Smiley et al., 2011; Smiley & Marshall, 2016). Therefore, this review article provides the latest information about the distribution and economic importance of main CNs on wheat, and the progress made in the identification and characterization of resistance genes from different sources and its utility against CCN and RLN, which will attract the attention of the scientific community and other relevant stakeholders.

Major Plant-Parasitic Nematodes of Wheat

Over 4100 species of PPNs have been characterized and documented (Decraemer & Hunt, 2006). PPNs are known to cause extensive damage to many plant species including wheat (Chen et al., 2017). Collectively, they cause annual estimated damage of $80–118 billion dollars to crops (Nicol et al., 2011). CCN attack wheat crop and cause significant damage in its production. Their damage is also documented on other cereal crops including barley (*Hordeum vulgare*) and oat (*Avena sativa*) (Amjad et al., 2019). In some wheat cultivating states of the USA (Idaho, Oregon, and Washington) an estimated 3.4 US$ million are lost annually (Smiley & Guiping, 2010). The yield losses caused by *H. avenae* in some wheat-growing fields could be in the range of 30 to 100% (Ibrahim et al., 1999; Nicol et al., 2004). Root-lesion nematodes are documented to cause in the range of 10 to 85% yield loss on wheat crop (Nicol & Rivoal, 2008; Smiley, 2010). Also, other losses of wheat are caused by the seed gall nematode (*Anguina tritici*), stem and bulb nematode (*Ditylenchus dipsaci*) (Tulek et al., 2015) and root-knot nematodes (*Meloidogyne* spp.) (Seid et al., 2015).

Distribution of Wheat Nematodes

Cereal cyst nematodes (CCNs)

The genus *Heterodera* is considered to be one of the oldest identified genera of PPN. In 1859, Schacht reported the first cyst-forming nematode on roots of sugar beet. Later, Schmidt erected *Heterodera* at the genus level and described it as *H. schachtii* in 1871 (Schmidt, 1871). Cyst nematodes parasitizing cereal crops were also reported in 1874 by Kühn in Germany. A species of the genus *Heterodera* parasitizing peas but differing from *H. schachtii* because it did not damage oats, hence, it was reported and named as *H. goettingiana* (Liebscher, 1892). With the documentation and recognition of the host-specificity of the nematode's cyst forming, more cyst nematode species could be identified and characterized. *Heterodera rostochiensis* which is one of the potato cyst nematode species was described from potatoes (Wollenweber, 1923), and *H. avenae* (oat cyst nematode) was reported from cereals (Wollenweber, 1924). Afterward, several nematodes forming cysts were detected on cereals and subsequently reported. These CCNs form a complex of numerous closely related species with a recognised global distribution on Poaceae family (Nicol et al., 2004; Nicol & Rivoal, 2008). *Heterodera avenae* was the first reported (Kühn, 1874), and then, the Mediterranean *H. latipons* (Franklin, 1969), followed by the north European *H. hordecalis* (Andersson, 1974), the eastern European *H. filipjevi* (Madzhidov, 1981), and others (Wouts et al., 1995). The cyst forming genus *Heterodera* contains 70 species including *H. avenae* group which is a complex of 12 known species and parasitizes cereals and grasses (McDonald & Nicol, 2005). However, *H. avenae, H. filipjevi* and *H. latipons* are considered the most economically important species in cereals worldwide (Nicol et al., 2007b) and each will be discussed briefly as follows.

Heterodera avenae

Heterodera avenae is an important species with global extensive distribution (Wen et al., 2019). It has a wide distribution in wheat-growing regions across Europe, Asia, Australia, the Mediterranean, South Africa, and North and South America (Smiley et al., 2017).

Heterodera latipons (Mediterranean cereal cyst nematode)

Heterodera latipons has been recorded from the Mediterranean region, Europe, Asia, and North Africa, but not in the USA (Smiley et al., 2017). This species has an extensive distribution and mainly in the Mediterranean region like Syria (Sikora & Oostendorp, 1986), Jordan (Yousef & Jacob, 1994), Lebanon (Greco et al., 2002) and Turkey (Rumpenhorst et al., 1996). Moreover, this species was found to be present in temperate climates of the former USSR (Subbotin et al., 1996), Iran (Tanha Maafi et al., 2007), and Europe: Czech Republic (Sabova et al., 1988), Bulgaria (Stoyanov, 1982), and United Kingdom (Anon, 2005) and Canada (Sewell, 1973).

Heterodera filipjevi (the rye cyst nematode)

Heterodera filipjevi has been reported in eastern and northern Europe, the former USSR (Balakhnina, 1989), in Turkey where it is the most dominant and widespread species of CCN (Rumpenhorst et al., 1996), the Mediterranean region, in Central and West Asia and North America (Smiley et al., 2017). In a very recent survey were conducted in Azerbaijan and Kazakhstan, *H. filipjevi* was found to be the dominant species in both countries (Dababat et al., 2019c, 2020). *Heterodera filipjevi* is probably the major destructive nematode in wheat and barley growing areas, particularly in semiarid areas where nematode damage increases under drought stress conditions (Rivoal & Cook, 1993; Nicol, 2002).

Root-lesion nematodes (RLN)

RLNs are ranked next to the CCNs in terms of its economic significance on wheat production systems (Castillo & Vovlas, 2007). Currently, eight species of the RLN (*P. brachyurus*, *P. coffeae*, *P. crenatus*, *P. neglectus*, *P. penetrans*, *P. pseudopratensis*, *P. thornei*, and *P. zeae*) are known to affect roots of cereals (Rivoal & Cook, 1993; Nicol et al., 2004) including wheat. *Pratylenchus thornei*, *P. neglectus*, *P. penetrans*, and *P. crenatus* are spreading widely and often co-exist in a single farm (Nicol et al., 2003; Smiley & Nicol, 2009). The spread of RLN species depends mainly on the presence of a suitable host plant that supports the reproduction and conducive environmental factors primarily temperature (Castillo & Vovlas, 2007). RLN species have been detected in 90% of dryland wheat-growing areas of the Pacific Northwest of the USA (Oregon, Washington, and Idaho), with a higher prevalence of *P. neglectus* and *P. thornei* (Smiley et al., 2004). *Pratylenchus thornei* is found to be a major nematode species in wheat production areas of the subtropical northern region of eastern Australia (Thompson et al., 2016). *Pratylenchus thornei* and *P. neglectus* are the most significant species of RLNs in damaging huge wheat-growing areas in the southern region of Australia (Vanstone et al., 2008). The occurrence of *P. thornei* and *P. neglectus* in diverse wheat-growing fields in Jordan has been documented (Al-Banna et al., 2015). *Pratylenchus thornei*, *P. neglectus*, and *P. scribneri* have also been characterized from wheat producing provinces of Isparta in Turkey (Sogut & Devran, 2011; Imren et al., 2020). *Pratylenchus neglectus*, *P. thornei, P. pseudopratensis* and *P. penetrans* have been recorded in wheat-growing fields of Iran (Ghaderi et al., 2010) where *P. thornei* and *P. neglectus* were found prevalent (Pourjam et al., 1999). In Morocco, RLN were found to be the most economically important group of PPN in diverse wheat-growing fields followed by the *H. avenae* group (Mokrini et al., 2017). The occurrence of *P. penetrans*, *P. thornei*, *P. pinguicaudatus* and *P. pseudocoffeae*, across different wheat-growing fields of Morocco has been reported

(Mokrini et al., 2016). Sikora (1988) characterized both *P. neglectus* and *P. penetrans* along with *P. thornei* on barley and wheat-growing areas in Northern Africa and all these as well as *P. zeae* in Western Asia. *Pratylenchus crenatus* Loof, 1960, *P. fallax* Seinhorst, *P. neglectus* Rensch, 1924, *P. penetrans* Cobb and *P. thornei* (Sher & Allen, 1953) were recorded in the east and southeast Anatolia (Yüksel, 1974). *Pratylenchus thornei* was documented in the Aegean and Thrace regions (Misirlioglu & Pehlivan, 2007). In wheat, *P. thornei* is the most studied and reported species (Nicol et al., 2000). It was found in Algeria, Australia, Canada, India, Israel, Italy, Mexico, Morocco, Pakistan, Syria, Turkey, and former Yugoslavia (Nicol et al., 2004).

Economic Importance of the Major Wheat Nematodes

Globally, PPNs are among the most encountered soil-borne biotic agents that attack wheat and cause significant yield loss of up to $157 billion per year (Bird & Kaloshian, 2003; Abad et al., 2008). Wheat yield losses caused by PPN were assessed at 11% annually in South Africa (Keetch, 1989). Among the PPNs, the CCNs and the RLNs are the most extensively studied genera and have been detected and recorded from many countries (Cook & Noel, 2002; Subbotin et al., 2010a; Dababat et al., 2015; Dababat & Fourie, 2018) affecting wheat production, the supply chain and the global food security.

Cereal cyst nematodes (CCNs)

CCNs are responsible for significant losses in cereal which have been documented and reported (Rivoal & Cook, 1993; Subbotin et al., 2010b; Imren et al., 2016, 2017; Mokrini et al., 2017; Fard et al., 2018) and most recently were intensively reviewed by Dababat and Fourie (2018). CCNs cause substantial economic losses especially in temperate and semi-arid regions of the world where the prevalent cropping system is monoculture (Rivoal & Cook, 1993). Among the CCNs, *H. avenae* is the most damaging on wheat production. This biotrophic phytopathogen parasitizes wheat and other cereals. Wheat yield losses caused by *H. avenae* are less than 10% under the climatic conditions of northern Europe, but the damage can be significantly higher and exceed 50% in hotter and drier climates (Imren & Elekcioğlu, 2014).

Economic losses due to *Heterodera* spp. on wheat have been documented across diverse regions of the world. The yield loss due to *H. avenae* has been reported 50% in wheat in Australia (Meagher, 1972), 40–92% in Saudi Arabia (Ibrahim et al. 1999), 40-50% in India (Mathur et al., 1980), and 40–50% in China (Peng et al., 2007), 26–96% in Tunisia (Namouchi-Kachouri et al., 2007), 50% in Israel (Mor et al., 1992) and 15-20% in Pakistan (Maqbool, 1988). Yield losses of 50% in wheat were recorded due to *H. avenae* and *H. filipjevi* in Norway (Holgado et al., 2004). Hassan et al. (2010) investigated the effect of *H. avenae* on both plant and nematode parameters such as plant growth, yield, and nematode multiplication in both durum and bread wheat cultivars under natural field conditions in Syria. Their finding indicated a 57% and 50% reduction of grain yields and 50% and 45% in straw yields in durum and bread wheat, respectively. In *H. avenae* heavily infested irrigated fields of the USA 50% yield reduction of wheat has been reported (Smiley et al., 1994). *Heterodera avenae* caused significant yield loss in different wheat cultivars, varying from 4 to 26% in Adana Province of Turkey (İmren & Elekcioğlu, 2014). A yield loss study was conducted in Rajasthan by Handa and Yadav (1991) was estimated up to 35% in wheat field.

Heterodera latipons is known to cause much lower damage when compared with *H. avenae* (Mor et al., 2008). However, the loss was found to be greatest in severe drought conditions and where the cropping system is monoculture (Philis, 1988, 1997). Durum wheat grain and straw losses due to *H. latipons* is higher in semi-arid regions of Syria (Scholz, 2001). *Heterodera latipons* was found significantly reducing the yield of winter wheat by 55%, spike height up to 36%, shoot dry weight by 48%, plant height up to 32% and root dry weight by 70% in Iran (Hajihasani et al., 2010a). This species was also found decreasing barley yield by 50% in Cyprus (Philis, 1988).

Yield losses in wheat due to *H. filipjevi* were reported in the range of 10-40% in China (Peng et al., 2007) and 40-92% in Saudi Arabia (Ibrahim et al., 1999). Smiley et al. (2005) reported *H. filipjevi* causing a 35% yield loss in spring wheat in Oregon, USA. Recently Fard et al. (2018) estimated yield losses in wheat yield ranging between 20% and 25% in Iran by *H. filipjevi*. Wheat grain yield loss caused by *H. filipjevi* occurred even at lowest initial population density (*Pi*) and stretched to 48% yield loss with a *Pi* of 20 (eggs +J2) per gram of soil in Iran (Hajihasani et al., 2010b). A study was led to assess the effect of *H. filipjevi* on selected wheat cultivars in the field in Turkey, and the results showed that there was a significant grain yield reduction (42%) in the studied wheat cultivars (Nicol et al., 2006). Similarly, a study to evaluate the impact of *H. avenae* on six spring wheat cultivars under naturally infested fields in Turkey showed that there was a significant yield reduction (25.7%) (İmren & Elekcioğlu, 2014). Sahin et al. (2008) reported that *H. filipjevi* was found infesting wheat fields in Haymana, Turkey where nematode population densities have reached (115 eggs and J2) per g soil. This may prove that *H. filipjevi* has great potential to cause damage and significant yield losses in wheat cultivation in Turkey and, this species caused yield loss in cultivars Seri-82 and Silverstar averaging 8.5% and 40.5% in two-year experiments (Imren et al., 2020), respectively. Fard et al. (2018) conducted field experiments to investigate the impact of *H. filipjevi* on three wheat cultivars and their results showed significant reductions in grain yield in the range of 19.5% and 27.8%. A micro plot experiment was executed to explore the impacts of *H. filipjevi* on the wheat yield of

the cultivar Sardari with different *Pi* values (Hajihassani et al., 2010). They reported that nematode density of 2.5 (eggs +J2) per gram of soil caused a yield reduction of 48%. Similarly, the damage caused by *H. filipjevi* to different wheat, barley, and triticale cultivars indicated a significant reduction in grain yield by 52% (40%–73%) in field conditions in Iran (Ahmadi et al., 2013). Yield losses of 42-50% on winter wheat were recorded due to *H. filipjevi* under rainfed conditions in Turkey (Nicol et al., 2006). In Iran, the yield loss caused by *H. filipjevi* was studied on winter wheat in the monoculture cropping system and resulted in 48% loss with a *Pi* density of 20 (eggs+J2) per gram of soil while the aerial shoot yield loss reported reaching 40% (Hajihasani et al., 2010a).

CCN has decreased yields in individual research trials or fields by 20% in Pakistan, 50% in Australia, 50% in Turkey, and 90% in Saudi Arabia (Dababat et al., 2015). Field infestation by CCN has caused 30-100% yield losses of wheat in different growing conditions (Bonfil et al., 2004; Nicol et al., 2007b). However, reports of crop losses at the magnitudes mentioned above do not precisely portray the scale of yield losses at a regional or national level since the documentation was mainly based on research plots located in infested areas of fields (Amjad et al., 2009). Furthermore, some early reports attributed to yield reduction due to *H. avenae* are recently reclassified as *H. australis*, *H. filipjevi*, *H. latipons*, or *H. sturhani*. However, numerous reports of regional or national wheat yield losses due to CCNs (Amjad et al., 2009).

Root-lesion nematodes (RLN)

Beside the CCNs, wheat is severely infected by several RLNs species of which *P. neglectus* and *P. thornei* are the destructive ones (Yu et al., 2012; Dababat et al., 2016). Wheat crop loss caused by *P. neglectus* can be as high as 85% (Smiley, 2010). *Pratylenchus neglectus* is found in Europe, Australia, and North America, which has not been studied much; nevertheless, 16-23% yield losses have been recorded in southern Australia by this species (Taylor et al., 1999). Studies in Oregon showed spring wheat yield losses of 36% associated with *P. neglectus* populations (Smiley et al., 2005). A 38-85% wheat yield losses due to *P. thornei* have been reported and documented in Australia, 50% in Oregon state of the USA, 12-37% in Mexico, and 70% in Israel (Nicol et al., 2004) and 32% in Turkey (Gozel & Elekçioglu, 2001). In Australia, *Pratylenchus thornei* is the most prevalent species of RLNs in wheat-growing areas and estimated to cause a loss of $33 million annually (Brennan et al., 1992). Several studies conducted on spring wheat reported yield losses of elsewhere in the world up to 32% by *P. neglectus*, and 69% caused by *P. thornei* (McDonald & Nicol, 2005; Thompson et al., 2008). Similar yield loss figures from this species have been stated for spring wheat in Oregon state (Smiley et al., 2005). Winter wheat yields were found reduced by 32% due to *P. thornei* in Colorado (Armstrong et al., 1993). Yield losses caused by *P. thornei* are assessed at 20% on wheat fields in Turkey (Toktay, 2008). *Pratylenchus penetrans* affects wheat crops yield by 10–19% in Canada (Nicol & Rivoal, 2008). Moreover, these two RLN species were always found to occur simultaneously (Dababat & Fourie, 2018).

Current Management Approaches

The population of CNs should be below the threshold levels to maintain acceptable quantities of production and reduce CNs damage. Damages caused by CNs can be most effectively controlled by integrating multiple strategies (Smiley & Nicol, 2009; Riley & Qi, 2015; Dababat & Fourie, 2018). Eradication of whole CNs from infested fields is impossible; however, the protection of non-infested fields is crucial. Once an infestation of a field has occurred, the goal of management is to scale back the density of pathogens below threshold levels that cause economic damage. CNs can be managed by integrated pest management, including field sanitation, providing adequate irrigation and fertilization, crop rotation, resistant cultivars, etc.

Field sanitation

Phytosanitary procedures are often useful at a limited level; however, the limitation of localities is extremely difficult or impractical. Managing the movement of soil from infested to non-infested areas is a key to reduce nematodes movement. Detecting of CNs in newly infested fields is very difficult and takes times until it is noticed. Cereal nematodes are distributed through various ways such as soil transported by tools, plant products, animals, water, and wind (Smiley et al., 1994; Dawabah & Al-Hazmi, 2007).

Crop rotation

Crop rotation aims to maintain a balance between the nematode populations and the frequency of cultivation. This is vital to ensure that sufficient time occurs after the nematode population increases on a susceptible/preferable host. This is a fundamental method to keep the CNs below the economic threshold for the next susceptible host. To implement the most effective crop rotation; the identity of the nematodes and the diversity of their hosts, the population dynamics, the degree of susceptibility of different hosts, and the relationship between the density of the nematodes and the lack of yield (crop tolerance) must be determined (Smiley et al., 2008). Damage by CNs is most prominent when the non-resistant host plants are grown. Dual combinations of resistant cultivars and non-cereals can efficiently control CCNs. The utilization of crop rotation, however, could be completely different for RLNs due to their polyphagous nature (Nicol & Rivoal, 2008). Therefore, a thorough understanding of the effectiveness of rotation is required for the successful implementation of crop rotation. Crop rotation, which includes broadleaf crops, corn, resistant wheat or barley or oat varieties, can significantly reduce

the density of cyst nematodes (Rivoal & Sarr, 1987; Fisher & Hancock, 1991; Smiley et al., 1994).

Host-Plant Resistance and Tolerance for Management of Cereal Nematodes

The cultivation of host plants having tolerance or resistance is an efficient approach to control CNs (Thompson et al., 2008; Vanstone et al., 2008; Dababat et al., 2015). Both resistance and tolerance of a host are genetically independent characteristics. Varieties that are tolerant or resistant to one nematode species may not necessarily offer resistance or tolerance to another nematode species (Rivoal et al., 2001; McDonald & Nicol, 2005; Smiley & Nicol, 2009; Dababat et al., 2019a, b). Resistance to nematode is considered as the capacity of host plants to inhibit the reproduction rate of nematode (Cook & Evans, 1987) (Table 1). Therefore, nematodes do not multiply, produce empty cysts, or reproduce poorly in resistant plants. Susceptibility is often genetically different from tolerance, where the later indicates the host's ability to resist a nematode attack i.e., the ability of the host to keep the potential of economic yield despite of the presence of nematodes (Stanton & Stirling, 1997; Smiley et al., 2008, 2017).

Table 1. Definition of tolerance and resistance to nematode infection (Dababat, 2019)

		Nematode reproduction	
		Low	High
Plant Yield	High	resistant/tolerant	susceptible/tolerant
	Low	resistant/intolerant	susceptible/intolerant

Plants are considered resistant to nematodes when the expression levels of host genes associated with pathogenicity provide inhibition or reduction of the reproduction rate (Stanton & Stirling, 1997). Resistance is a conclusion of a change in the equilibrium of the nematode-host reaction and complex processes in the host-parasite interactions (Stanton & Stirling, 1997). The hypersensitive reaction to the nematode is the most common phenomenon of resistance, especially to sedentary endoparasites, and results in incompatible responses, which don't allow the nematode to feed. Less common mechanisms involved in resistance are plants not being able to attract nematodes, preventing eggs from hatching, the formation of toxins by plants, and resistance to penetration of nematodes (Smiley et al., 2008). Broader sustainability, however, may help solve problems with resistant varieties. Additionally, plant resistance is not present in many important varieties, and their effectiveness is generally limited to a few species/pathotypes of nematodes. This may result in varieties that tend to select virulent nematodes or related biotypes found in field populations (Whitehead 1998; Gheysen et al., 1996). The use of resistant varieties reduces the risk intensity for the next successive wheat, barley, or oats. Even if reproduction is restrained, infective juveniles often penetrate and damage the roots of resistant hosts, resulting in reducing the yield. Ideally, tolerance should be combined with resistance which is the best control option (Smiley et al., 2017). One of the advantages of using a tolerant host as a control strategy is that it does not exert selective pressure on the nematode as resistance. However, if the host is also susceptible, the nematode population can multiply, ultimately exceeding limits and causing yield loss (Rivoal et al., 2003; Smiley & Nicol, 2009; Dababat & Fourie, 2018). So far, mechanisms involved in the tolerance of nematodes and other plant parasites are not well understood. Several proposed mechanisms, including, for example, plant growth that goes beyond what is necessary to obtain economic benefits, growth compensating for damage caused by nematodes and growth due to lack of response to the presence of nematodes. Tolerant plants can produce a lower number of galled tissues than intolerant plants, which allows them to maintain photosynthesis for normal growth.

Marker-assisted selection (MAS) for CN resistance in wheat is commonly used to describe stable germplasm in selection programs around the world. The combination of greenhouse testing and marker-based reproduction has been used worldwide in studies aimed at reducing invasion and loss due to disease (Ogbonnaya et al., 2009). The marker-based selection strategy consists of two steps: pre-propagation for characterization of resistance sources and development of linked markers, followed by different sources (e.g. *Cre3* on chromosome 2BL, *Cre3* on chromosome 2DL and *Cre8* on chromosome 6B), specific PCR for tracking each gene using markers (Ogbonnaya et al., 2009).

Resistance to RLN is a quantitative trait, whereas resistance to CCN is genetically controlled by one gene (Mokrini et al., 2018). In the case of MAS, molecular markers have been developed to identify genes and quantitative indications of resistance in seedlings. Molecular markers have been successfully applied to identify resistance genes against CN in barley and wheat (Barr et al., 1998; Eagles et al., 2001; Ogbonnaya et al., 2001a, b; Barloy et al., 2007). Marker-based selection is used to improve genetic resistance, but effective resistance genes are not yet available in all cultures nor effective for all pathotypes. An extensive set of experiments using a tube, pot, or trial run screening determines whether the wheat, barley, oat, and triticale lines demonstrate resistance to a CN population. However, pathogenic phenotyping is tedious, time-consuming and takes mostly a full season to complete. By developing dominant or co-dominant molecular markers and testing to determine resistance to the CN populations, leaf samples from small seedlings can be taken to determine the presence of resistance genes in 1-2 days, saving time and money. Therefore, selection based on the CN resistance markers in wheat is often used to describe resistance germplasm in breeding programs around the world. The combination of phenotyping and genotyping is used worldwide in

Table 2. Principal sources of genes used to breed wheat for resistance to the cereal cyst nematode *Heterodera* species, unless stated otherwise

Cereal Species	Cultivar or Line	Origin	Resistance Gene(s)[a, b]	Response to Pathotypes[b,c]	Use in Cultivars
Wheat					
Triticum aestivum	Loros, AUS10894	? [d]	*Cre1*[e] (formerly *Ccn1*), on chromosome 2BL	pR to several pathotypes	NW Europe, Australia; NW USA - under evaluation
	Katyil	Australia	*Ccn1*	S, India	Australia
	Festiguay	Australia	*Cre8* (formerly *CreF)* on chromosome 7L? Recent analysis suggests 6B	pR to *Ha13*	Australia
	AUS4930 = Iraq 48[i]	Iraq	possibly identical genetic location as *Cre1*; also resistance to Pt	R to several pathotypes and *Heterodera* species and Pt	Australia, France, CIMMYT- under evaluation
	Molineux	Australia	chromosome 1B (14% resistance)	R to Ha13	Australia
	Raj MR1 (Raj Molya Rodhak1)	landrace from Nigde, Turkey AUS 15854 x J-24	one dominant gene	R only to some populations of *H. avenae*, appears S to Indian *H. filipjevi*	Released cultivar in northern India in 2002
Triticum durum	Psathias 7654, 7655, Sansome, Khapli	?	?	S to some pathotypes, pR to others	
Triticale and rye					
Triticosecale	T701-4-6	Australia	*CreR* on chromosome 6RL	R to Ha13	Australia
	Drira (=Ningadhu)	Australia	?	R to Ha13	Australia
	Tahara	Australia	?	R to Ha13	
	Salvo	Poland	?		UK
Secale cereale	R173 Family		*CreR* on chromosome 6RL	R to Ha13	Australia
Wild grass relatives of wheat					
Aegilops tauschii	CPI 110813	Central Asia	*Cre4* on chromosome 2DL	R to Ha13	Australian synthetic hexaploid lines
Aegilops tauschii	AUS18913	?	*Cre3* on chromosome 2DL	R to Ha13	Australian advanced breeding lines
Aegilops peregrina (= *Ae. variabilis)*	1		*Cre(3S)* with *Rkn2* on chromosome 3S; *CreX,* not yet located		
Aegilops longissima	18	?	?	R to four French pathotypes and *Meloidogyne naasi*	France
Aegilops geniculata	79; MZ1, MZ61, MZ77, MZ124	?		R and pR to several pathotypes	France – under evaluation
Aegilops triuncialis	TR-353	?	*Cre7 (*formerly *CreAet)*	R and pR to several pathotypes	France – under evaluation
Aegilops ventricosa	VPM 1		*Cre5* (formerly *CreX*), on chromosome 2AS	R to several pathotypes	Spain – under evaluation
	11; AP-1, H-93-8		*Cre2* (formerly *CreX*) on genome N[v]		
	11; AP-1, H-93-8, H-93-35		*Cre6,* on chromosome 5N		
Madison			*Cre9*	https://pubmed.ncbi.nlm.nih.gov/31433275/	

[a] [b] *Sources*: Reviews and references in Rivoal and Cook (1993), Cook and Rivoal (1998), McDonald and Nicol (2005), and Nicol and Rivoal (2007). Characterized single-gene resistance to cereal cyst nematode. [c] R = resistant, pR = partially resistant, S = susceptible. [d] ? = no published scientific studies conducted. [e] Marker implemented in commercial breeding program – refer to Ogbonnaya *et al.* (2001b).

studies aimed at reducing invasion and disease loss (Ogbonnaya et al., 2009).

Cereal cyst nematodes (CCN)

Host resistance remains the most profitable and easiest to apply management process. However, it can only be used by farmers if the varieties have a tolerance (yield performance) that is comparable to other prevalently grown wheat varieties. The reasons for resistance in CCN populations around the world were compared, analysed, and gene localization and mapping were performed where possible (Table 2) (Rivoal et al., 2001; Dababat & Fourie, 2018). More details about this section are under in the subtitle “Source of Resistance”.

Root-lesion nematodes (RLN)

The use of resistant varieties is the most promising and economical tool to lower radically the RLN populations. More details about this part are under in subtitle sources of resistance for RLN.

Chemical control

Repeated use of chemical nematicides has been shown to control CCNs in wheat (Smiley et al., 1994; Dababat et al., 2014; Fard et al., 2015; Riley & Qi, 2015). The chemicals used to control nematodes can be divided into two main groups as contact or systemic nematicides and fumigants. Their costs usually limit the use of nematicides in intensive agriculture in which high-quality plants are produced instead of the crops that need a larger area for cultivation such as grains (Smiley et al., 2017). Before 1980, several fumigants such as methyl bromide, dichloropropene, chloropicrin, dibromochloropropane (DBCP), ethylene dibromide (EDB), metham-sodium, and dazomet were frequently used against nematodes worldwide. These chemicals are not selective and affect all soil organisms including weeds, bacteria, fungi, and other invertebrates as well as nematodes (Dababat & Fourie, 2018). Fumigants for nematode control show their effect by diffusion from the pore space of the gas phase and the water film that surrounds the soil particles. The movement and effectiveness of the fumigants are affected by the amount of organic matter that is not degraded in the soil, temperature, and texture of the soil. Substances with such fumigating properties quickly kill nematodes and then dissolve in the soil. All fumigants are generally phytotoxic and should be used as a pre-sowing treatment as excellent nematicides, which offer a high level of nematode control (Smiley & Nicol, 2009; Smiley et al., 2008). There are two types of non-volatile nematicides, carbamates and organophosphates. Carbamates that are highly toxic to birds, mammals, fish, and humans include aldicarb, carbofuran, and oxamyl as most commonly used chemicals to control nematodes. Organophosphate and carbamate nematicides are usually applied to the upper few centimetres of the soil using planting equipment and are distributed downward with the movement of water. All non-volatile nematicides are highly toxic to mammals but are rarely phytotoxic at the concentrations used for field control (Smiley et al., 2017). Aldicarb is a pre-plant nematicide, very effective, and the most widely used nematicide. However, due to the increased level of microbial inactivation, aldicarb became ineffective after repeated use to control CCN in irrigated wheat areas in Saudi Arabia (Dawabah et al., 2015). In areas affected by aldicarb, repeated application of oxamyl to leaves causes a decrease in the density of *H. avenae* and an increase in the wheat yield. However, after two alfalfa products, the yield of wheat was not affected positively by oxamyl (Dawabah et al., 2015). Thus, oxamyl was proposed for regular use as part of a well-defined integrated CCN management system, which includes crop rotation, soil fertility, and sanitation management in the field, and rotating of the chemical composition of nematicides. The most common organophosphates are cadusaphos, terbufos, ethoprophos, and fenamiphos. Carbamates and organophosphates tend to have a hemostatic effect due to the action of nemastatic in action rather than nematoxic, which causes nematodes to become incapacitated, preventing egg hatching, reducing mobility, inhibiting feeding, and retarding development (Stirling et al., 1992). Nematodes are effective in the soil for a limited period (usually 2-6 weeks) and therefore tend to resume normal activity if chemicals are lost. Since nematodes need to be identified in a relatively short time frame, non-volatile nematicides are well suited for use in annual crops. However, to control nematodes in perennial plants, it is necessary to apply them more than one time in a year. The development of strategies for using these materials in drip irrigation systems expands their use in such situations (Stirling et al., 1992).

Biological control

Various bacteria, fungi, and invertebrates are known to prey or parasitize, and soils that suppress nematodes biologically have been identified (Davies et al., 1991). Economic constraints and environmental and safety concerns associated with early generation nematicides, however, have eliminated them on behalf of most farmers (Starr et al., 2007). Also, economical and effective biological nematicides for the treatment of CNs are currently not available in rain-fed farming systems. Efforts are underway to develop effective control of nematicides. Abamectin (a mixture of B1a and B1b of avermectin) has been rated as a seed treatment for CNs treatment, but its advantages were not important to marginal wheat fields infected with cyst nematode (*H. avenae*) in Israel (Oka et al., 2009) and the United States (Smiley et al., 2012). However, when higher abamectin levels are used in seed furrows in China, grain yield has increased significantly (Zhang et al., 2017).

Bacillus firmus spores used in biological seed treatment in the United States also had little effect on grain yield and density after harvesting the cyst nematode (*H. avenae*) (Smiley et al., 2012). The leaf application of a broad-spectrum insecticide/nematicide, spirometramat, which has mobility in both xylem and phloem (Safferling, 2008), reduced the density up to 78% after harvesting the cyst nematode (*H. avenae*), but did not improve the yield of summer wheat or influence the number of root galls on roots (Smiley et al., 2011). Cui et al. (2017) noted that treating winter wheat seeds with a mixture of fipronil plus chlorpyrifos, or either methylene (bis)thiocyanate plus thiamethoxam increases wheat yield and reduces the number of cysts in soils infected with the Chinese cyst nematodes populations (*H. filipjevi* and *H. avenae*). More research is still needed to investigate the various combinations of these nematicides and other modern nematicides. Dababat et al. (2014) and Tian et al. (2007) examined reports that cyst nematode populations were reduced to densities not economically important by bacterial and fungal parasites of J2s and eggs. These organisms reduced the number of cysts formed, the ability to shed eggs, and the viability of J2s. However, there is currently no evidence that this phenomenon can be effectively

manipulated as a practical management strategy in most parts of the world. It seems that there is no successful commercial practice for CN biological suppression.

Other management strategies

As a rule, it is not possible to set the time for growing plants. Hence, one of the most effective control options in cool and temperate regions where nematodes hatch in the spring is to plant winter crops in the fall and deepen in the roots to maximize hatching speed. Although this strategy is not as effective as rotation or genetic resistance, it can be a useful part of an integrated CCN pest management approach. The maximum yield loss from CN is seen when water or nutrients are limited by the maximum potential for plant growth at any time during the growing season. Thus, crop damage is minimized by ensuring optimal plant nutrition and, if possible, supplemental water during intervals of drought (Fard et al., 2015; Singh et al., 2009). Also, the CCN population can be reduced by planting susceptible hosts as traps until the main hatching period, thereby facilitating plant root invasion, which then dies before newly developing white females can produce viable eggs (Stone, 1961).

Source of Resistance

Source of Resistance to Cereal cyst nematodes (CCN)

Utilization of resistant and tolerant wheat germplasm to combat CCNs is an eco-friendly and cost-effective strategy (Trudgill, 1991). However, such resistant cultivars should be continuously grown on a particular area which leads to lower negligible population densities (Ali et al., 2019). Sowing of these resistant varieties primarily results in lower reproduction rates in the invading nematode species (Cui et al., 2016). This is a well-known fact that almost all of the reported resistances in commercial cultivars against CCN are introgressions of a single dominant gene (Rivoal et al., 2001; Nicol, 2002; Nicol et al., 2003; McDonald & Nicol, 2005; Nicol & Rivoal, 2008; Smiley & Nicol, 2009).

The very early discovery of gene harbouring resistance to *H. avenae* was carried out from barley from Sweden in 1920, nevertheless, this gene was completely characterized in 1961 (Andersen, 1961). This was followed by a huge amount of work aimed at the development of CCN resistant cultivars by several scientists in the coming decades (Smiley et al., 2017). Later, a *H. avenae* resistance locus was reported in barley on chromosome 2H (*Ha2* locus) and confirmed by using RFLP markers (Kretschmer et al., 1997). The same molecular technique was used by Barr et al. (1998) which led to the mapping of *Ha4* locus on chromosome 5H in barley.

Many sources of resistance to different diseases in bread wheat are derived from wild relatives of wheat via conventional breeding programmes (Ogbonnaya et al., 2001a). Overall the resistance sources to *H. avenae* comprise 9 resistance loci/genes (formerly called: Cereal root eelworm: Cre) which were introgressed into bread wheat from other *Triticum* spp. and *Aegilops* (Barloy et al., 2007). These loci range from *Cre1* to *Cre9* in addition to *CreR* and *CreV*. *Cre1*, *Cre8* and *Cre9* originate from *T. aestivum* itself, *Cre2*, *Cre5* and *Cre6* from *Aegilops ventricosa, Cre3* and *Cre4* from *Aegilops tauschii*; *Cre7* from *Aegilops triuncialis* L. However, *Dasypium villosum* L. is the source for *CreV* while *CreR* was reported from rye (Barloy et al., 2007). All these loci are well documented and mapped on the wheat chromosomes; however, *CreX* and *CreY* are sourced from *Ae. variabilis* are not well characterized for their inheritance mode and their locations are still unknown on the wheat chromosomes (Barloy et al., 2007).

Different studies showed that the *Cre1* locus is relatively more responsive to *H. avenae* population from Europe, North America and North Africa (Table 2) whereas this locus is less responsive to Australian and Asian populations of CCNs (Rivoal et al., 2001; Mokabli et al., 2002). In addition to different responsiveness of *Cre* loci in different countries and continents, the effectiveness of *Cre* genes varies in response to different species of CCNs (Ali et al., 2019). For instance, *Cre1* gene was active in response to Turkish populations of *H. filipjevi*. While, *Cre3* lost its effectiveness to Turkish populations of *H. filipjevi* (Smiley et al., 2017). However, the *Cre3* gene was found to be active against the Australian populations of *H. avenae* (Vanstone et al., 2008), but this resistance was lost to *H. avenae* populations from Europe (de Majnik et al., 2003; Safari et al., 2005). On the other hand, *Cre2* and *Cre4* loci originated from *Aegilops* spp. demonstrated expansive degree of resistance against numerous *Heterodera* spp. and their various pathotypes (Nicol et al., 2001). CIMMYT coordinates the work of the international root resistance nursery, which includes seven of the *Cre* genes to determine the value of these genes globally. Quantitative trait loci (QTLs) associated with CCN resistance were also linked to chromosomes 1A, 1D, 4D, 5A, 5B, 5D, 6A, 6B, 7A, and 7D (Mulki et al., 2013; Dababat et al., 2016). Eleven DArT markers have also been reported (Dababat et al., 2016). These areas of resistance could soon become molecular tools for wheat-growing programs.

Nonetheless, the resistance against CNs must be coupled with a tolerance response to achieve sustainable productivity in wheat (Brown, 1987). Tolerant cultivars have been demonstrated to show better grain yield with a considerable degree of nematode control (Smiley, 2009). As compared to resistance response, under field conditions, tolerance response is assessed by relating grain yield of nematicide treated control plot and a nematode infested untreated plot (Brown, 1987; Smiley & Marshall, 2016). This displays real-time assessment of wheat cultivars against nematode infestations which are

primarily based on grain yield and development of nematodes on the plant roots.

The tolerance trait is mainly a result of specific attributes of physiological response and root growth of the plants in response to nematode infestation (Stanton & Fisher, 1988; Volkmar, 1990). During the establishment of syncytia, in case of cereal cyst nematodes, root growth and development is extremely restricted because of the abbreviation of root and sometimes proliferation in the form of adventitious roots, and root depth is largely decreased. This leads to unavailability of water and water-soluble nutrients that leads to a decrease in the overall productivity of the plants. Most of the time, the resistance response is negatively correlated with grain yield and during the absence of nematodes, susceptible cultivars tend to have a higher yield as compared to resistant wheat varieties (Wilson et al., 1983). This is why, the farmers sometimes do not prefer resistant varieties due to lower grain yields as compared to susceptible ones in non-infested soils (Rivoal & Cook, 1993). Conversely, a coupling of tolerance and resistance responses in wheat cultivars may lead to enhanced per unit production and profitability (Smiley et al., 2017).

Sources of Resistance to Root-lesion nematodes (RLN)

Deployment of host resistance is considered one of the most ideal and economical strategies to reduce the detrimental effects caused by the RLN populations (Castillo et al., 1998). In contrast to the inherited single gene for resistance to CCNs, resistance to RLNs is largely quantitative. This kind of horizontal resistance is good in a sense that there is always some degree of resistance available, however, on the other hand, due to additive effects from several genes; it is sometimes difficult to develop effective resistance. In addition to native species of wheat from middle East, wild relatives of wheat i.e., *Aegilops* species are important sources of resistance against *P. thornei* (Hollaway et al., 2000; Nombela & Romero 1999; Nicol et al., 1999, 2001, 2003; Sheedy et al., 2008; Thompson & Haak, 1997; Thompson et al., 1999; Tokay et al., 2006; Zwart et al., 2004, 2005). Several accessions showed a resistance to both *P. thornei* and *P. neglectus* (Dababat et al., 2016, 2019; Nicol et al., 2007a; Sheedy et al., 2007; Zwart et al., 2005). By using double-resistance sources in commercial varieties, farmers no longer need to identify *Pratylenchus* at the species level before deciding on a sustainable variety.

Numerous sources of resistance to RLN have been described in the wheat germplasm Table 3 (Taylor et al., 2000; Thompson & Haak, 1997; Toktay et al., 2012). For instance, resistant resources to *P. thornei* in wheat (Thompson et al., 1999, 2009; Vanstone et al., 1998) and *P. neglectus* (Thompson et al., 1999) are already known. Bread wheat line "GS50a" was significantly reported to be the first source of resistance to *P. thornei* from Australia, which it was primarily selected from the cultivar "Ghatcher" (Thompson & Clewett, 1986). Ten times lower RLN reproduction was found on GS50a in comparison with the local control (Thompson et al., 1999). A reasonable number (i.e., 274 accessions) of Iranian landraces of wheat were assessed for resistance to RLN and 25 of those accessions showed more resistance than that of GS50a line (Sheedy & Thompson 2009). Similarly, Thompson et al. (2009) performed the screening experiment with wheat accessions from North Africa and West Asian regions and found some additional sources resistant to *P. thornei*.

Mapping of QTLs and phenotypic identification of resistance sources have been largely used to identify

Table 3. Principal sources of genes used to breed wheat for resistance to root-lesion nematodes *Pratylenchus neglectus* (*Pn*) and *P. thornei* (*Pt*)

Cereal Species	Cultivar or Line	Origin	Resistance Gene(s)[a,b]	Response to Pathotypes[b,c]	Use in Cultivars
Triticum aestivum	GS50a	Australia - reselection from cv. Gatcher	Major QTL mapped to 6D		Australia
	AUS4930=Iraq 48	Iraq	QTLs mapped to 1B, 2B, and 6D	R to *Pt* but also portrays R to Ha	Australia, CIMMYT – under investigation
	Reselection of Excalibur	Australian cv. Excalibur	QTL mapped to 7AL	R to *Pn* (*Rlnn1*)	Australia, CIMMYT
	Croc_1/*Ae. tausch*. (224)//Opata	Primary synthetic	QTLs mapped to 1B and 3B	R to *Pt*	CIMMYT
	CPI133872	Primary synthetic	QTLS mapped to 2B, 4D, 6A, and 6D	R to *Pt* and *Pn*	Australia
	W-7984 x Opata 85		QTLs mapped to 2B and 6D	R to *Pt*	Australia
	AUS4926	Middle eastern landrace	QTLS mapped to 1B, 2B, 3B, and 6D	R to *Pt*	Australia
	AUS13124	Middle eastern landrace	QTLs mapped to 2B, 3B, 6D, and 7A	R to *Pt*	Australia
Aegilops tauschii	CPI 110872			R to *Pt* and *Pn*	
Aegilops geniculata	MZ10, MZ61, MZ96, MZ144	Middle East and West Asia		pR to Pt., several also portray R to *Ha*	

[a] *Sources*: Reviews and references in Rivoal and Cook (1993), Cook and Rivoal (1998), McDonald and Nicol (2005), and Nicol and Rivoal (2007).
[b] Characterized QTLs associated with multigene resistance to root-lesion nematodes.
[c] R = resistant, pR = partially resistant, *Ha* = *Heterodera avenae.*

resistance sources against RLNs. The QTLs linked to resistance against *P. thornei* resistance are mapped on different chromosomes of bread wheat i.e., 1B, 2B, 3B, 4D, 6D, and 7A (Schmidt et al., 2005; Toktay et al., 2006; Zwart et al., 2005). *Rlnn1* locus which is located on the 7A chromosome offers substantial resistance to *P. neglectus* at the seedling stage (Williams et al., 2002). According to Williams et al. (2002), *Rlnn1* originated from Australian variety 'Excalibur', has been identified and validated for its better degree of resistance against *P. neglectus*. Similarly, another locus conferring resistance to *P. neglectus* has been characterized and identified on the 4D chromosome (Zwart et al., 2005). The relationships between resistance reactions and markers were adequately constant to show *Pratylenchus* in wheat the possibility of using the marker selection to increase resistance. *Rlnn1* marker has been successfully used following this strategy and is actively implemented as part of international wheat breeding programs in CIMMYT at a global level and in Australia (Williams et al., 2002).

P. thornei is the most investigated species of RLN in the resistance studies because it is the most dominant RLN species around the globe. As compared to *P. thornei, P. neglectus* is less investigated for screening and resistance studies. However, combined resistance studies and development of resistance to both RLN species is desirable as these species are present in the same field with mixed populations (Thompson et al., 2010). Moreover, Smiley & Nicol (2009) reported that resistance and tolerance response to *P. thornei* and *P. neglectus* is genetically independent because of the observation that wheat cultivars tolerant or resistant to one species did not show a similar response against the other species.

Multiplication rate of RLN is high on susceptible varieties of wheat that leads to a reduction in growth and grain yield; however, a resistant cultivar supports lower nematode reproduction leading to lower yield losses. By contrast, a cultivar with tolerance response is still able to yield better even when the population densities of RLN are higher in the field (Thompson et al., 1999). The early sources with superior tolerance response to *P. thornei* i.e., cultivars like Baxter, Pelsart and Sunvale (Brennan et al., 1994; Ellison et al., 1995; Thompson et al., 1999) were identified through targeted screening to curtail the damaging effects of RLN. Thompson et al. (1995) reported that the above-mentioned tolerant lines led to a 30% yield enhancement over the commercial varieties of wheat being grown in the field at that particular time. This necessitates the development of cultivars in which the resistance response should be combined with tolerance to combat RLN. The most comprehensive investigation regarding the breeding of tolerant and resistant lines to *Pratylenchus* spp. is done by Australian scientists. This research demonstrated cultivation of a tolerant wheat cultivar after nematicide treatment and/or in the nematode-free fields is one of the better options for RLN management (Thompson et al., 2008).

Phenotypic identification of resistance was used in combination with molecular biology to investigate the genetic control and localization of resistance genes and to identify resistance markers. The locations of QTL associated with resistance to *P. thornei* have been identified on chromosomes 1B, 2B, 3B, 4D, 6D, and 7A (Schmidt et al., 2005; Zwart et al., 2005, 2006; Toktay et al., 2006; Dababat et al., 2016). A molecular marker may describe the presence of the *Rlnn1* gene on the 7A chromosome, which offers resistance to *P. neglectus* in seedlings (Williams et al., 2002). Another resistance gene for *P. neglectus* was characterized on the 4D chromosome (Zwart et al., 2005). The relationships between resistance reactions and markers were adequately constant to show *Pratylenchus* in wheat the possibility of using the marker selection to increase resistance. This process is actively implemented as part of international wheat breeding programs in Australia and CIMMYT using the *Rlnn1* marker (Williams et al., 2002).

Transgenic resources

Conventional breeding for enhancement of nematode resistance or tolerance often demands large scale screening of germplasm accessions to locate resistance loci and validation of these loci using appropriate molecular markers (Ali et al., 2019). However, the employment of transgenic strategies provides a potential alternative to conventional breeding programmes. The transgenic approach provides both heterologous and homologous transfer of validated natural as well as synthetic resistance genes. There are several transgenic strategies available for the developing transgenic wheat resistance to both CCN and RLN. We have recently provided a detailed review regarding different transgenic methods to develop nematode resistance in plants (Ali et al., 2017a).

This review indicates the use of host induced gene silencing approach to suppress effector genes which are vital for the establishment of nematodes on plant roots (Ali et al., 2017a, 2017b). Although this strategy is not employed for the development of nematode resistance against CCN and RLN, except a study where 4 genes involved different physiological process of *H. avenae* were silenced through in vitro silencing (Gantasala et al., 2015). The researchers reported 26%, 60% and 71%, 26%, and 60% decrease in the number of eggs and females because of silencing of genes encoding intron binding protein, epsin, and polyadenylate binding protein, respectively. Conversely, eggs and females were increased up to 25% due to the silencing of nuclear hormone receptor. Likewise, the *H. avenae* annexin like protein (Ha-annexin) was supposed to be active for the suppression of basal defence responses (Chen et al., 2015). HIGS of Ha-annexin led to lower nematode development of *H. avenae* in wheat. Most recently, genes coding for two *H. avenae* venom allergen-like

effector proteins (HaVAP1 and HaVAP2) involved in the suppression of programmed cell death were silenced and characterized (Luo et al., 2018).

Transgenic expression of protease inhibitors (PIs) like cystatins, trypsin inhibitors and serine proteinase inhibitors are also good resources for the development of CCN and RLN resistance in wheat. PIN2, a serine proteinase inhibitor from potato was transformed into the durum wheat which enhanced *H. avenae* resistance in wheat (Vishnudasan et al., 2005). Other genetic sources include nematicidal proteins like Bt-toxins and lectins (Ali et al., 2017b). Similarly, use of anti-invading chemodisruptive peptides and a combination of proteinase inhibitors and chemodisruptive peptides could be a potential approach to increase resistance against CCN and RLN in wheat (Ali et al., 2017b, 2019).

Conclusion and Future Remarks

Global food security largely depends on sustainable wheat production to feed the ever-increasing population. CCN and RLN are a serious threat to global wheat production and must be managed through appropriate combating strategies. The access to genomic resources like genome sequence and re-sequence information, and wheat worldwide germplasm collection centres including wild wheat germplasm collections may provide unique opportunities for wheat genetic improvement. Moreover, the presence of several genome-wide association studies and genome editing technologies could also help for further improvement to enhance CCNs and RLNs resistance in wheat. This article provides the latest information regarding the progress made in the identification and characterization of resistance genes from different sources and its utility against CCNs and RLNs, which will attract the attention of the scientific community and other relevant stakeholders.

This particularly emphasizes the enhancement of resistant wheat germplasm using both conventional as well as modern approaches. In addition to marker-assisted selection (MAS) and common selection approaches, use of genotyping by sequencing (GBS) followed by genome-wide association studies (GWAS) could be used for the development of nematode resistance in wheat (Pariyar et al., 2016b). Likewise, assessment to complete genome sequences of different cereal nematodes could lead to identifying novel effector encoding genes for manipulation via host-induced gene silencing (HIGS) approach (Consortium, 2012; Mayer et al., 2014). Moreover, the recently completed sequencing of the wheat genome has generated a huge opportunity that could be exploited to study and understand molecular wheat-nematode interactions leading to the development of nematode resistant wheat (Appels et al., 2018).

Moreover, transcriptome studies for both wheat and barley in response to CCN and RLN infection could provide information and could be manipulated through modification of gene expression in wheat roots aimed at the development of nematode resistance (Ali et al., 2019). Transgenic expression of rice cystatins proteinase inhibitor has recently been carried out in several crops i.e. banana, tomato, plantain, and potato (Ali et al., 2017a). Cystatins are readily digestible in the human digestive system and their use might have limited biosafety and environmental issues (Tripathi et al., 2015). These PIs could be potential candidates to be used for transgenic expression to incorporate CCN and RLN resistance in wheat. Combination of various techniques and resources like HIGS, use of proteinase inhibitors and root-specific expression of anti-invading nematode repellent peptides could amplify nematode resistance in wheat (Ali et al., 2017a). More recently, application of CRISPR/Cas9 system could be used for targeted genome editing for enhancement of CCN and RLN resistance in bread wheat (Kumar & Jain, 2015).

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors Contribution

AD and SA conceived and designed the review. AD, SA, MAA, FT, TP, and MI wrote and edit the review. All authors contributed to the article and approved the submitted version.

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