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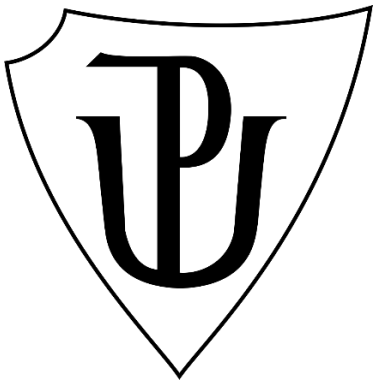

Efekt prospěšné bakterie *Pseudomonas fluorescens* na buněčná a vývojová stadia tolice vojtěšky (*Medicago sativa*, L.) během abiotického stresu

Bakalářská práce

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Vedoucí práce:	**Jasim Basheer, PhD.**
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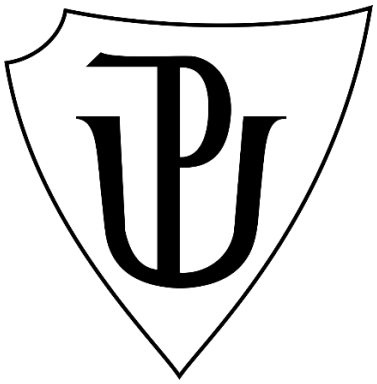

Effect of beneficial bacterium *Pseudomonas fluorescens* on the cellular and developmental stages of alfalfa (*Medicago sativa*, L.) during abiotic stress

Bachelor thesis

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Study form:	Attendant
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Year:	2022

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Abstrakt

Vojtěška setá (*Medicago sativa*, L.) je víceletá pícnina, která se pro svou bohatou výživovou hodnotu hojně využívá ve výživě hospodářských zvířat jako siláž. Vojtěška jako luskovina vytváří uzliny, zvláštní orgány, které umožňují soužití symbiotických mikroorganismů s hostitelskou rostlinou. Bakterie podporující růst rostlin (PGPB) jsou skupinou mikroorganismů, které žijí v symbióze s rostlinami. PGPB podporují růst rostlin buď přímo, nebo nepřímo produkcí sloučenin, jako jsou auxiny nebo cytokiny. Mezi tyto bakterie patří i *Pseudomonas fluorescens*. Je to aerobní, tyčinkovitá gramnegativní bakterie, která kromě užitečných sloučenin produkuje fluoreskující žlutozelené siderofory. Solný stres představuje pro zemědělství vážný problém způsobený osmotickým stresem, nižším výnosem biomasy nebo pomalejším klíčením a růstem rostlin. Aby se rostliny během stresu ochránily, musí mít vyvinuté mechanismy, které tyto negativní účinky překonávají. Mitogenem aktivované proteinkinázy (MAPK) jsou proteiny signální kaskády, které přenášejí mezibuněčné signály a poskytují adekvátní buněčnou odpověď. Principem je fosforylace MAPKKK receptorem, MAPKKK fosforyluje MAPKK a MAPKK fosforyluje MAPK. MAPK aktivují biomolekuly, které zprostředkovávají odpověď v rostlinách. V této práci jsme se pokusili studovat úlohu SIMKK (mitogenem aktivované proteinkinázy kinázy indukované solným stresem) a účinek PGPB během solného stresu na rostlinách vojtěšky seté. *Medicago sativa*, L. cv. Regen-SY (WT – divoký typ) a *Medicago sativa* L. cv. Regen-SY SIMKK RNAi (kde je SIMKK downregulována) byly použity pro hodnocení solného stresu a jeho zmírnění pomocí *Ps. fluorescens* pomocí fenotypizace a mikroskopické analýzy.

Klíčová slova	*Medicago sativa,* L.*; Pseudomonas fluorescens* R562, růst kořenů, MAPK, solný stres, mitigace, SIMKK, spinning disk mikroskop
Počet stran	34
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Abstract

Alfalfa (*Medicago sativa*, L.) is a perennial forage legume that is widely use in livestock nutrition as a silage for its rich nutritional value. As a legume, alfalfa forms nodules, special organs that allow symbiotic microorganisms coexistence with the plant host. Plant Growth Promoting Bacteria (PGPB) are a group of microorganisms that forms relationship with plants. PGPB supports the growth of plants either by direct or indirect means by producing compounds like auxins or cytokines of which *Pseudomonas fluorescens* is a part of. It is an aerobic, rod-shaped gram-negative bacterium which produces fluorescent yellowish green siderophores along the compounds. Salt stress poses a serious problem to agriculture caused by osmotic stress, lower biomass yield or slower germination and growth of the plants. To protect themselves during the stress, plant must have developed mechanisms to overcome these negative effects. Mitogen-activated protein kinases (MAPK) are signal cascade proteins which transduce extracellular signals and give adequate cell response. The principle is the phosphorylation of MAPKKK by receptor, MAPKKK phosphorylates MAPKK and MAPKK phosphorylates MAPK. MAPK activate the biomolecules that mediate the response in plants. In this thesis, we tried to study the role of SIMKK (salt stress-induced mitogen-activated protein kinase kinase) and the effect of PGPB during salt induced stress on alfalfa (*Medicago sativa* L.) plants. *Medicago sativa* L. cv. Regen-SY (WT) and *Medicago sativa*, L. cv. Regen-SY *SIMKK RNAi* (where the SIMKK is downregulated) were used for the evaluation of salt stress and its mitigation using *P. fluorescens* by phenotyping and microscopy analysis.

Keywords	*Medicago sativa*, L.; *Pseudomonas fluorescens* R562, root growth, MAPK, salt stress, stress mitigation, SIMKK, spinning disk microscope

Number of pages	34
Number of appendices	0
Language	English

AIMS OF THE THESIS

- Determine the effect of *Pseudomonas fluorescens*, a bacterium belonging to the Plant Growth Promoting Bacteria, on cellular and developmental stages of alfalfa (*Medicago sativa,* L.).
- Determine the phenotypic changes in two lines of alfalfa (*Medicago sativa* L.): *Medicago sativa* Regen-SY (wild type) and *Medicago sativa* Regen-SY SIMKK RNAi (line with downregulation in SIMKK expression)

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1. INTRODUCTION

Alfalfa (*Medicago sativa,* L.) is a perennial forage legume belonging to *Fabaceae* family. The origin of alfalfa is considered in Asia, spreading towards the Iberian Peninsula. Alfalfa is recognizable for its typical violet corolla and trifoliate leaves. Alfalfa is used worldwide mainly as a silage in animal husbandry for its nutritional value, being rich in proteins, important macro- and microelements, vitamins, and antioxidants. Alfalfa is also sold as a nutritional supplement, and it has become a part of human food as a "green food". Being a legume, alfalfa forms nodules on roots to allow symbiosis with nitrogen-fixing bacteria. Alfalfa had become a tool in studying symbiotic relations between plant and microorganisms. In laboratory conditions, alfalfa can be grown by somatic embryogenesis, which is the process of growing a whole new plant from a small part of the explant.

Salt stress is posing a great problem in agriculture. The consequences of saline soils are irreversible, for that the plants cannot survive for a long time during stressful conditions caused by higher concentrations of salt. The effect of salt stress is osmotic stress which cause certain cell death, along with lower biomass yield and retardation in growth. Plants are sessile organisms and due to this, they had to develop protective mechanisms. Mitogen-activated protein kinases (MAPKs) are a type of protein kinase that are specific to the aminoacids serine and threonine. MAPKs are involved in directing cellular responses to a diverse array of stimuli like osmotic stress, heat shock and proinflammatory cytokines. They regulate cell functions including proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis. These kinases play an unmistakeable part in cell signal transduction. The mechanism of MAPKs is the phosphorylation of each other: MAPKKK phosphorylates, and therefore activates, MAPKK, which then phosphorylates MAPK. MAPKKK itself is activated by a receptor found on the plasma membrane of the cell. MAPK is transferred into cell nucleus or to another site of the cytoplasm, and activates other biomolecules, such as enzymes, other kinases or cytoskeletal proteins and thus respond to extracellular signal, for example molecules that are the result of extraordinary event, such as stress. A special type of MAPK has been discovered in alfalfa during salt stress – salt stress-induced mitogen-activated protein kinase. SIMKK, an analog to MAPKK, enhances the activation of SIMK

and the plant can respond to salt stress. SIMK is located mainly in nucleus but can be distributed into growing hair root tips.

In this study, the effect of PGPB (Plant growth Promoting bacteria) is tested for its ability to help and mitigate abiotic stress caused by salt and their association with SIMKK. PGPB have beneficial effect on plant growth and health in general by producing compounds that are important for plant life. PGPB not only produce life-dependant compounds for the plant, but also help to protect them from pathogens. *P. fluorescens*, a gram-negative, rod-shaped bacterium belongs to the PGPB group. It colonizes the surface of the plant root. Not only does *P. fluorescence* produce compounds important for the plant, such as auxin indole-3-acetic acid, but also produces siderophore pseudobacin, which is yellowish green and fluorescent pigment. Siderophores bind iron ions and can therefore deprive the pathogenic fungi or bacteria from getting the iron ions that are needed for their survival.

The further research on PGPB effects during salt stress might pose a usable biological tool in overcoming salinity that threatens the nourishment of millions of people.

2. CURRENT STATE OF THE PROBLEM

2.1 Alfalfa (*Medicago sativa*, L.)

Alfalfa (*Medicago sativa* L.) is a perennial legume belonging to the *Fabaceae* family (Kirschner & Štěpánek 1995). The centre of origin of the genus *Medicago* is north-western Iran, north-eastern Turkey, and the Caucasus, spreading west to the Mediterranean area and the Iberian Peninsula, East to China, North to Russia and South towards the Arabian Peninsula (Quiros & Bauchan 1988).

2.1.1 Overview of alfalfa anatomy

The length of the stem of Alfalfa ranges from 30 to 100 centimetres. Petioles are relatively short, measuring 0.5-1.5 centimetres; trifoliate leaves are 1-3 cm long, up to 1 cm wide. The inflorescence is elliptic, reaches up to 3 cm in length, from 8 to 25 flowers. The calix is 5-9 mm long, the corolla measures 8-12 mm and has a typical violet colour. The seedpod is indehiscent and curled, with usually 3-6 seeds. The seeds are mostly ellipsoid, brownish yellow, 2.5-2.8 mm long and 1.3-1.5 mm wide (Kirschner & Štěpánek 1995; Schauer 2005). Figure 1 shows a botanical drawing by French botanist Amédée Masclef, published in 1891 in Atlas des plantes de France (Atlas of plants of France).

Figure 1 - Anatomical drawing of alfalfa published in Atlas des plantes de France (1891) by Amédée Masclef. Source of image: Wikimedia Commons.

2.1.2 Nitrogen fixation and root nodule formation in alfalfa

Alfalfa gains nitrogen from both the soil and the atmosphere. The atmospheric nitrogen, which is present in its molecular form (N_2), is made available to the plant with the help of symbiotic fixation (Heichel, 1981). Alfalfa forms nodules on the roots, which is characteristic feature of many other legumes (Boisson-Dernier *et al*., 2001). A nodule is a special organ that allows symbiotic relationship between the plant and microorganisms (Rhizobium) which in turn produces available nitrogen which can be utilised by the host plant (Brewin, 1991). The formation of nodules begins with initiation of bacterial attachment to the root hairs, initiation and elongation of the infection thread. With the help of infection thread the bacteria from root hair reaches the inner layers of root cortex where the bacteria are released. These bacterial cells induce the cortical cells to multiply which results in the formation of nodules on the surface of the roots. The bacterial cells will also multiply and colonize inside the multiplying host cells. As the available space in the host cells is completely filled, the bacterial cells become dormant and are called bacteroids. The bacteroids usually occur inside the cytoplasm in groups. Each group of bacteroids is surrounded by a membrane called peribacteroid membrane. The space surrounded by peribacteroid membrane is called peribacteroid space. A red pigment called leghaemoglobin is present outside the peribacteroid space in the cytosol of the nodule cells which are responsible for the maintenance of the conditions required for the bacteriods to fix nitrogen to the available form to the plants (Rolfe & Gresshoff, 1988; Sprent, 1989; Brewin, 1991; Truchet *et al*, 1989; Brewin, 1991).

2.1.3 Use of alfalfa

Alfalfa is primarily used as a silage in animal husbandry due to its nutritional value (Radović *et al*., 2009). It contains high amounts of proteins, important macro-elements and micro-elements, vitamins, and antioxidants. Nevertheless, alfalfa is on the rise in being used as a valuable part of human food. As the market demand for so-called “green food” grows, scientists have performed many experiments to find out the full potential of alfalfa as a part of human nutrition (Mielmann 2013).

2.1.4 Plant tissue culture

Plant tissue culture is a set of methods that aims to produce new whole plants from an aseptic culture of plant cells, tissues, or organs in the laboratory conditions. It is necessary to use nutrient solutions for the correct plant growth. The environment, in

which the plant cultures are developed must be monitored and regulated (Loyola-Vargas & Vázquez-Flota, 2006).

Plants can regenerate in two main ways: either via somatic embryogenesis or organogenesis. Somatic embryogenesis covers two types of embryogenesis: direct and indirect embryogenesis. In both ways, the main goal remains the same: to form new somatic embryos, from which a new plant will grow. In direct embryogenesis, there is no dedifferentiation stage, and embryonic cell formation can be completed directly from the surface of explants, in which minimal genetic reprogramming is involved (eg. plant development directly from internodes). Whereas in indirect embryogenesis is a multistep regeneration process including callus formation, somatic embryo formation, maturation, and conversion which requires major reprogramming and thus can result in somaclonal variations. The callus is capable of production of somatic embryos. For the correct callus development, auxins (such as IAA) are required. When auxins are removed from the medium, embryo formation begins. During organogenesis new organs (shoots or roots) are formed, which depends on the presence of auxin or cytokinin in the medium (Bhatia & Bera, 2015).

Several types of plant tissue cultures are being used in practice, such as apical meristem culture, axillary bud culture, callus culture, suspension culture, protoplast culture, embryo culture, and anther culture (Kumar & Loh, 2012; Gaikwad *et al*., 2017). Plant material for apical meristem culture is usually harvested from actively growing shoot tops under the node leaves, where the apical meristem is located. For performing axillary bud culture, a meristem tissue from axillary buds located on mature stalk must be harvested. These two methods are advantageous in eliminating plant viruses (Cheong *et al*., 2012). Callus culture is based on production of undifferentiated mass of cells that is induced as a recovery tissue after wounding. The main advantage of this technique is the fact that nearly any part of the plant can be used for callus formation. In this method, it is required to supply the nutrient medium with growth hormones such as auxin and cytokinin (Efferth, 2019). Another method used in plant cell culture is plant cell suspension culture where a crumbly callus must be used. It is because the plant cells in liquid medium tend to aggregate, therefore the more friable the callus the better. Stirring is also necessary to achieve non-aggregated cells (Godoy-Hernández & Vázquez-Flota, 2012). In protoplast culture, cells without cell wall are used. These cells can be obtained by treating the plant material (mostly leaf cells) with cell wall degrading enzymes, such as cellulase and

pectinase. Viable protoplasts are determined by fluorescein diacetate (FDA) staining method (Pan *et al.*, 2006). The FDA method's principle is in the ability of esterases to hydrolyse the stain, resulting in fluorescein, which has the fluorescent ability, therefore is an indicator of live cells (Grimm *et al.*, 2013). In embryo culture, seed and ovule embryos are used to be cultivated in a nutrient medium. These embryos then grow into a whole new plant. This technique is used to overcome seed dormancy and to produce haploid plants and allows to grow rare species of plants (Hussain *et al.*, 2012). Anther culture method results in producing haploid plants. This method can be used in breeding programs and offer a good approach for genetic analyses in biotechnology (Irikova *et al.*, 2011).

2.2 Abiotic stress

Abiotic stress has a negative impact on living organisms like salinity, drought, heat, or cold. These factors cause mass crop loss around the world (Ben-Ari & Lavi, 2012).

2.2.1 Soil salinity and salt stress

Soil salinity poses a serious problem in terms of crop production in agriculture. According to the Global Map of Salt-Affected Soils launched by the Food and Agriculture Organization of the United Nations (FAO) in October 2021, 8.7% of the global soil are affected by salinity. The term salinity refers to a soil condition that contains high concentrations (electrical conductivity is 4 $dS \cdot m^{-1}$ or higher) of soluble salts (Munns & Tester, 2008). The electrical conductivity of 4 $dS \cdot m^{-1}$ is approximately comparable to 40 $mmol \cdot l^{-1}$ of sodium chloride (Al-shareef & Tester, 2019).

Increased salinity in soil causes plants to germinate and grow slower, water and nutrient uptake is decreased (Akbarimoghaddam *et al*., 2011; Jouyban, 2012). If exposed to higher concentration of salt, the plant roots lose water due to hyperosmotic effect of the salty soil, which could eventually cause wilting or senescence of the plant (Munns, 2002). Leaf stomates tend to close due osmotic stress, which results in decrease of photosynthetic activity (Munns & Tester, 2008). Sodium cations are being absorbed by the plants from hypersaline soil, and compete with potassium cations (Schachtman & Liu, 1999). Potassium is an essential element involved in neutralization of both inorganic and organic anions, pH homeostasis and regulates cell osmotic pressure; therefore, when being displaced by sodium cations, the cell turgor is disrupted (Nieves-Cordones *et al*., 2016).

2.3 Mitogen-activated protein kinases

In eukaryotes, mitogen-activated protein kinases (MAPK) are among the most important proteins in terms of signal transduction (Song, 2019). Mitogen-activated protein kinases activation begins with phosphorylation at threonine and tyrosine residues of TXY activation motif (Kiegerl et al., 2000). The MAPKs are in fact cascade proteins, which means there are (at least) three serine/threonine kinases that phosphorylate each other; in this case, a MAP kinase kinase kinase (MAPKKK/MAP3Ks/MEKKs) when initially activated by a signal phosphorylates MAP kinase kinase (MAPKK/MAP2Ks/MEKs), and the MAPKK then phosphorylates MAP kinase (MAPK/MPK) (Jagodzik el al., 2018). The MAPKKK phosphorylates MAPKK (downstream) on two serine or threonine residues motif. MAPKK, when active, shows a dual-specificity *ie*. it phosphorylates MAPK on the threonine and tyrosine residues in TXY motif (Cristina et al., 2010; Hettenhausen et al., 2014; Jagodzik et al., 2018). MAPKs are then transferred to the site of need either in cytoplasm or in nucleus, where they activate other kinases, cytoskeletal proteins, enzymes or transcription factors, (Khokhlatchev et al., 1998; Cristina et al., 2010). Scheme in Figure 2 depicts the cascade of MAPKs.

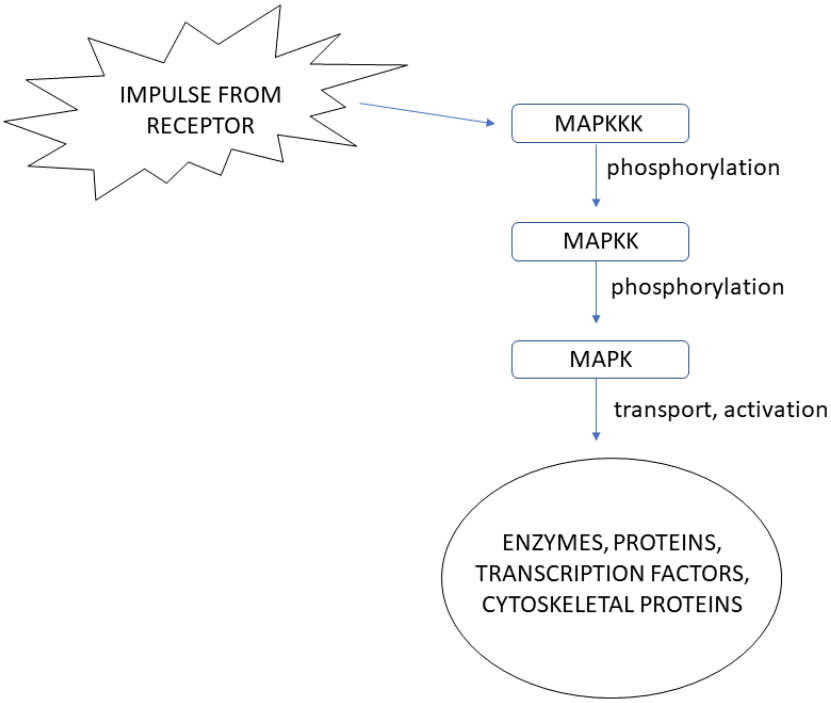

Figure 2 - Scheme of MAPK cascade. (Inspired by: Jagodzik *et al.*, 2018)

2.3.1 Salt-stress induced mitogen-activated protein kinases

Salt stress-induced mitogen-activated protein kinases (SIMK) are a special group of MAPKs that was found in alfalfa cells during the treatment with salt stress. SIMK is activated by sodium chloride, potassium chloride and sorbitol (Munnik *et al*., 1999). Later it was found that SIMK is activated specifically by SIMKK. SIMKK therefore enhances the activation of SIMK by salt stress (Kiegerl *et al*., 2000). SIMK is mainly located in the nucleus in epidermal cells, and during the formation of root hair, the active form of SIMK was redistributed into growing tips of root hairs. By observing the overexpression of gain-of-function SIMK induced rapid root hair tip growth (Šamaj *et al*., 2002). In recent study, it was found that overexpression of SIMKK promotes root hair growth, increases the capacity to form nodules and increases biomass yield. In contrast, downregulation of SIMKK and SIMK expression causes reduction in root hair growth, lower capacity of forming nodules and lower biomass yield (Hrbáčková *et al*., 2020).

2.4 Plant Growth Promoting Rhizobacteria

Plant Growth Promoting Rhizobacteria (PGPR) are a group of microorganisms, especially bacteria, which has the ability to enhance the growth and yield of plants via producing various substances that act as biofertilizers (Singh, 2013). The vast majority of rhizobacteria occupy a soil layer close to the plant root system called the rhizosphere (Hiltner, 1904). This is due to root secretion of substances that are beneficial and nutritious for rhizobacteria. The bacteria must be rhizosphere competent in order to have beneficial effects on plant. The example of rhizosphere competence is successful competition with other microbes within the root for root nutrients and for colonization of the root (Lugtenberg & Kamilova, 2009).

While being known mainly for the increase of plant yield and promote plant growth, PGPR are also known for different beneficial things, for example bioremediation of soils containing heavy metals by temporarily remove toxic heavy metal compounds, the degradation of xenobiotic compounds, improving soil structure by excreting polysaccharides, the synthesis of 1-aminocyclopropane-1-carboxylate (ACC) deaminase to decrease the level of ethylene in roots that was induced by stress, provide nitrogen to plant via fixation or producing auxins and cytokinins (Glick, 2004; 2010; Etesami & Maheshwari, 2018). Moreover, PGPR are known for mitigation of salt stress in plants (Paul and Lade, 2014; Shrivastava and Kumar, 2015; Qin et al., 2016).

2.4.1 Pseudomonas fluorescens

Pseudomonas fluorescens is an aerobic, gram-negative, rod-shaped bacterium with multiple flagella. It can be found mainly in the soil and water. The optimal temperature range for cultivating *P. fluorescens* is between 25-30 °C. As a part of PGPR, it colonizes the root surface and lives with beneficial relationship with the plant. Ps. fluorescens produces indole-3-acetic acid (IAA), an important phytohormone belonging to auxin family. Aside IAA, siderophores are also produced by *P. fluorescens*. These compounds bind iron, which could prevent getting other organisms, for example pathogenic fungi, the iron that is needed for their proliferation. The siderophore, namely pseudobactin, is a yellow-green, fluorescent pigment (David et al., 2018).

3. EXPERIMENTAL PART

3.1 Materials

3.1.1 Chemicals

- 1000x Gamborg vitamin – Duchefa Biochemie (The Netherlands)
- 1000x Nitsch & Nitsch vitamin - Duchefa Biochemie (The Netherlands)
- 2,4-dichlorophenoxyacetic acid (2,4-D) - Duchefa Biochemie (The Netherlands)
- Amino acid stock
- Boric acid (H_3BO_4) – Sigma-Aldrich (USA)
- Calcium chloride ($CaCl_2$) – Sigma-Aldrich (USA)
- Copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) – Duchefa Biochemie (The Netherlands)
- Disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$)
- Ethanol (96 %) - Tereos TTD (Czech Republic)
- (Ethylenedinitrilo)tetraacetatoferrate (FeEDTA) – Sigma-Aldrich (USA)
- Gamborg B5 basal salt mixture – Duchefa Biochemie (The Netherlands)
- Gellan Gum – Alfa Aesar (USA)
- Kinetin (KIN) – Duchefa Biochemie (The Netherlands)
- L-Proline – Sigma-Aldrich (USA)
- Luria-Bertani Broth (Miller) – Sigma-Aldrich (USA)
- Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) – AppliChem (Germany)
- Manganese (II) sulfate - Sigma-Aldrich (USA)
- Microagar – Duchefa Biochemie (The Netherlands)
- MilliQ H_2O - Simplicity® UV Water Purification System, Merck (USA)
- Murashige & Skoog basal salt mixture – Duchefa Biochemie (The Netherlands)
- Myoinositol – Duchefa Biochemie (The Netherlands)
- Potassium dihydrogen phosphate (KH_2PO_4) – Sigma-Aldrich (USA)
- Potassium hydroxide (KOH) – Sigma-Aldrich (USA)
- Potassium nitrate (KNO_3) – Sigma-Aldrich (USA)
- Propidium iodide – Sigma-Aldrich (USA)
- Sodium chloride (NaCl) – Sigma-Aldrich (USA)
- Sodium hypochlorite (NaOCl) – Honeywell/Fluka™ (USA)
- Sodium molybdate dihydrate ($Na_2MoO4 \cdot 2H_2O$) – Sigma-Aldrich (USA)

- Sucrose – Sigma-Aldrich (USA)
- Tween-20 – Sigma-Aldrich (USA)
- Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) – Sigma-Aldrich (USA)

3.1.2 Laboratory instruments and equipment

- Analytical scales – Radwag XA 110/2X (Czech Republic)
- Cell Observer SD Axio Observer Z1 Spinning Disk Microscope - Carl Zeiss (Germany)
- Computer - Lenovo (China)
- Confocal laser scanning microscope LSM880 with Airyscan - Carl Zeiss (Germany)
- Incubator (for autoclaved media) - Memmert (Germany)
- Laboratory incubator – Verkon (Czech Republic)
- Laminar flow cabinet – Merci Biohazard (Czech Republic)
- Loop Sterilizer - Solaris, Schuett Biotech (Germany)
- Magnetic stirrer - IKA COMBIMAG PRO (Drehzahl Electronic)
- pH meter – EUTECH Instruments PC 2700 (USA)
- Refrigerators - Electrolux Space Plus (Sweden), Liebherr Mediline (Germany)
- Scales – BEL Engineering (Italy)
- Scanning machine - GE Image Scanner III - General Electric (USA)
- Spectrophotometer - Smart Spec™ plus (Bio-Rad; USA)
- Vortex mixer – Vortex Genie 2 – Scientific Industries (USA)
- Axio Zoom Microscope with fluorescence attachment – Carl Zeiss (Germany)

3.1.3 Software

- EPSON scan, LAS V 4.0
- ImageJ/Fiji 1.46
- GraphPad Prism 9.3.1
- Microsoft Office 365
- Zen 3.5 (blue edition)
- Zen (2012) (black edition)

3.1.4 Laboratory tools

- Automatic pipettes

- Beaker
- Cover glass
- Double sided tape
- Falcon tubes (50ml)
- Glass slides
- Measuring cylinder
- Micropore tape
- Parafilm
- Petri dishes
- Pincers
- Scissors
- Small tube stand
- Spectrophotometer cuvette
- Spoon spatulas
- Sterile razor
- Transparent tape
- Weighing boats

3.1.5 Biological material

- Alfalfa (*Medicago sativa*, L.) cv. Regen-SY
- Alfalfa (*Medicago sativa*, L.) cv. Regen-SY (*SIMKK RNAi* downregulated line)
- *Pseudomonas fluorescens* strain R562 (GFP Tagged)

3.2 Methods

3.2.1 Indirect somatic embryogenesis

3.2.1.1 Leaves sterilization and calli formation

Somatic embryogenesis was done according to protocol by Samac & Austin-Phillips (Samac & Austin-Phillips, 2006) with slight modifications (Figure 3). For somatic embryogenesis, initially the leaves from *M. sativa* wild type and *SIMKK RNAi* lines were selected and surface-sterilized. Healthy matured leaves were harvested from the plant into 50 ml tubes with tap water for preventing dehydration. For sterilisation, tap water from the tubes were discarded and leaves were treated with 70% ethanol (v/v) for 30 seconds. After carefully discarding the ethanol, the leaves were washed with 0,1% Tween 20 solution (v/v) for 10 minutes. The leaves were then sterilised with 1%

(available chlorine) sodium hypochlorite (v/v) prepared in 0,1% Tween 20 (v/v) for 1.5 minutes followed by washing in sterile MilliQ water for 3 times.

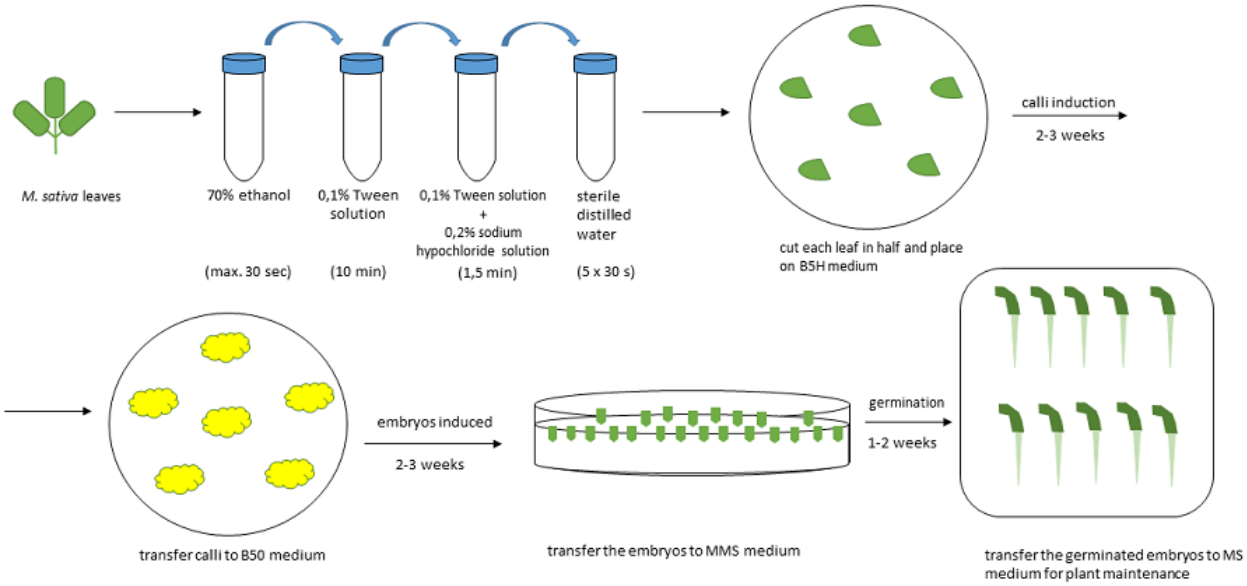

Figure 3 Scheme of somatic embryogenesis used for cultivating plant materials used in the study

After sterilization of the leaves, they were cut in half using a sterile scalpel. The leaves cuts were immediately placed on plates containing B5-H medium (Tables 1, 2 and 3). The labeled petri dishes were then sealed and incubated in a phyto-chamber for 3 to 4 weeks at 22-24 °C, 60-80 $mE \cdot m^{-2} \cdot s^{-1}$, 16 hours light/8 hours dark for the formation of calli. The plates were checked periodically for any contaminations.

Table 1 Composition of B5H medium (pH 5.7)

Composition	Quantity
Gamborg's B5 basal salt mixture	3.1 $g \cdot l^{-1}$
Potassium nitrate	0.5 $g \cdot l^{-1}$
Magnesium sulfate heptahydrate	0.25 $g \cdot l^{-1}$
Proline	0.5 $g \cdot l^{-1}$
Sucrose	30 $g \cdot l^{-1}$
Gellan Gum	4.5 $g \cdot^{l-1}$
1000x Gamborg's vitamin solution	1.0 $ml \cdot l^{-1}$
Stock amino acids	30 $ml \cdot l^{-1}$
2,4-D solution	1.0 $ml \cdot l^{-1}$
Kinetin solution	1.0 $ml \cdot l^{-1}$

Table 2 Composition of solution of stock amino acids

Composition	Quantity
Glutamine	26.6 $g \cdot l^{-1}$
Serine	3.32 $g \cdot l^{-1}$
Adenine	0.016 $g \cdot l^{-1}$
L-glutathione	0.332 $g \cdot l^{-1}$

Table 3 Concentrations of stock growth regulators

Composition	Quantity
2,4-D	1.0 mg.l-1
Kinetin	0.1 mg.l-1

After 3 weeks, the calli formed on B5-H media were carefully transferred to B5-0 medium (Tables 2 and 4) under aseptic conditions for the induction of embryos. The incubation was continued under the same conditions as mentioned above for 2-3 weeks.

Table 4 Composition of B50 medium (pH 5.7)

Composition	Quantity
Gamborg's B5 basal salt mixture	3.1 $g \cdot l^{-1}$
Potassium nitrate	0.5 $g \cdot l^{-1}$
Magnesium sulfate heptahydrate	0.25 $g \cdot l^{-1}$
Proline	0.5 $g \cdot l^{-1}$
Sucrose	30 $g \cdot l^{-1}$
Gellan Gum	4.5 $g \cdot^{l-1}$
1000x Gamborg's vitamin solution	1.0 $ml \cdot l^{-1}$
Stock amino acids	30 $ml \cdot l^{-1}$

After 3 weeks of incubation, the embryos formed on B5-0 were transferred on to MMS medium (Table 5) for root development. Incubation was carried out in the same conditions as mentioned above for 1-2 weeks.

Table 5 Composition of MMS medium (pH 5.7)

Composition	Quantity
Murashige & Skoog basal salt mixture	4.3 $g \cdot l^{-1}$
Myoinositol	0.1 $g \cdot l^{-1}$
Sucrose	30 $g \cdot l^{-1}$
Gellan Gum	4.5 $g \cdot l^{-1}$
1000x Nitsch & Nitsch vitamin solution	1 $ml \cdot l^{-1}$

The embryos in the MMS were monitored regularly for the root development and once the roots were developed, they were transferred on Murashige & Skoog (MS) medium (Table 6) for plant development. The plantlets with equal development were moved to Fåhraeus medium (Table 7) for 3 to 4 days for acclimatisation.

Table 6 Composition of MS medium (pH 5.7)

Composition	Quantity
Murashige & Skoog basal salt mixture	4.3 $g \cdot l^{-1}$
Sucrose	30 $g \cdot l^{-1}$
Gellan Gum	4.5 $g \cdot l^{-1}$

Table 7 Composition of Fåhraeus medium (pH 6.5)

Composition	Stock solution	Quantity
$MgSO_4 \cdot 7H_2O$	1.232 $g \cdot 10\ ml^{-1}$	1 $ml \cdot l^{-1}$
KH_2PO_4	0.953 $g \cdot 10\ ml^{-1}$	1 $ml \cdot l^{-1}$
$Na_2HPO_4 \cdot 2H_2O$	0.712 $g \cdot 10\ ml^{-1}$	2 $ml \cdot l^{-1}$
$FeSO_4 \cdot 7H_2O$	0.056 $g \cdot 10\ ml^{-1}$	2.5 $ml \cdot l^{-1}$
Fe EDTA	0.15 $g \cdot 10\ ml^{-1}$	2.5 $ml \cdot l^{-1}$
$MnSO_4 \cdot H_2O$	0.01 $g \cdot 10\ ml^{-1}$	100 $\mu l \cdot l^{-1}$
$CuSO_4 \cdot 5H_2O$	0.051 $g \cdot 10\ ml^{-1}$	100 $\mu l \cdot l^{-1}$
$ZnSO_4 \cdot 7H_2O$	0.028 $g \cdot 10\ ml^{-1}$	100 $\mu l \cdot l^{-1}$
H_3BO_3	0.01 $g \cdot 10\ ml^{-1}$	100 $\mu l \cdot l^{-1}$
$Na_2Mo_4 \cdot 2H_2O$	0.011 $g \cdot 10\ ml^{-1}$	100 $\mu l \cdot l^{-1}$
Microagar	…	11 $g \cdot l^{-1}$
$CaCl_2$	1.1098 $g \cdot 10\ ml^{-1}$	100 $\mu l \cdot l^{-1}$
0 $mmol \cdot l^{-1}$ NaCl	…	0 $g \cdot l^{-1}$
50 $mmol \cdot l^{-1}$ NaCl	…	2.922 $g \cdot l^{-1}$
75 $mmol \cdot l^{-1}$ NaCl	…	4.383 $g \cdot l^{-1}$

3.2.3 Experiment initiation and phenotyping of the selected lines

After acclimatisation, the plantlets were moved to plates with Fåhraeus medium supplemented with different concentrations of sodium chloride (0; 50; 75 $mmol \cdot l^{-1}$; Table 7) under aseptic conditions. The plates were incubated in phytotron under the same conditions as mentioned above.

3.2.3.1 Preparation of bacterial culture

The bacterial culture was prepared using Luria-Bertani medium (LB). For this, 1 ml of frozen bacteria glycerol stock was defrosted to room temperature. The aliquot was

then added in 25 ml of liquid LB medium in 50 ml tube. The culture was incubated in a shaker (180 rpm) at 28 °C for 24 – 48 hrs. After incubation, the culture was centrifuged at 7000 rpm at 20 °C for 7 minutes. The supernatant was carefully discarded, and pellet was dissolved in 2 ml of liquid Fåhraeus medium using repeated pipetting. After the resuspension, the final volume was adjusted to 25 ml with liquid Fåhraeus medium. The culture was again incubated in a shaker (180 rpm) at 28 °C for 4 hours. After 4hrs, the optical density (OD_{600}) of bacterial culture was measured in a spectrophotometer using sterile liquid Fåhraeus medium as a blank. Based on the initial OD obtained, the bacterial cultures were diluted using liquid Fåhraeus medium to obtain an OD ~ 0.2. The culture with an OD of ~ 0.2 was used for the treatment of the prepared plantlets. The treatment with *Pseudomonas fluorescens* was done the 3rd day after transferring to the media containing salt. Uninoculated liquid Fåhraeus medium was used in mock treatment. The growth of the plantlets was scanned using scanner once per day for 10 days since the beginning of plantlet cultivation before the bacterial treatment.

3.2.4 Visualisation of root colonization

The selected bacteria-treated plantlets from salt treatments were analysed using Zoom microscope (Carl Zeiss, Germany) with fluorescent attachment with 32x magnification. The images were processed using ZEN 2010 software.

3.2.5 Viability determination by propidium iodide using Spinning Disk Microscope

For microscopic analysis, the roots of both control and treated samples were used. For this, the roots from the respective control and treatments were placed in a plate containing propidium iodide (1 $mg.ml^{-1}$ final concentration) prepared using sterile liquid Fåhraeus medium for 8 min. After staining, the 1.5 cm long root tips were cut and placed between slide and coverslip prepared using double sided tape. The cassette containing the stained roots were observed under spinning disk microscope (Carl Zeiss, Germany) equipped with Plan-Apochromat 20×/0.8 NA (Carl Zeiss, Germany) using a 2-channel excitation at 534 nm (for PI) and 488 nm (for GFP tagged bacteria). Image post-processing was done using ZEN 2010 software.

4. RESULTS AND DISCUSSION

4.1 Somatic embryogenesis

Somatic embryogenesis was used for cultivating the plant materials to be used in all the experiment in the thesis. For this, sterile plant leaves were incubated on plates containing B5-H medium for callus followed by B5-0 for embryo development and MMS for root development. The different developmental stages during this process of plant formation were documented using Zoom microscope (Carl Zeiss, Germany) and images were processed using ZEN 2010 software. The representative pictures of both *M.sativa* wild type and mutant *SIMKK RNAi* during indirect somatic embryogenesis are shown in Figure 3. From the observation, the wild type leaves were slightly bigger than the *M.sativa SIMKK RNAi* leaf. However, the amount of callus, embryos produced, and size of the embryo were more or less equal both the lines (Figure 4).

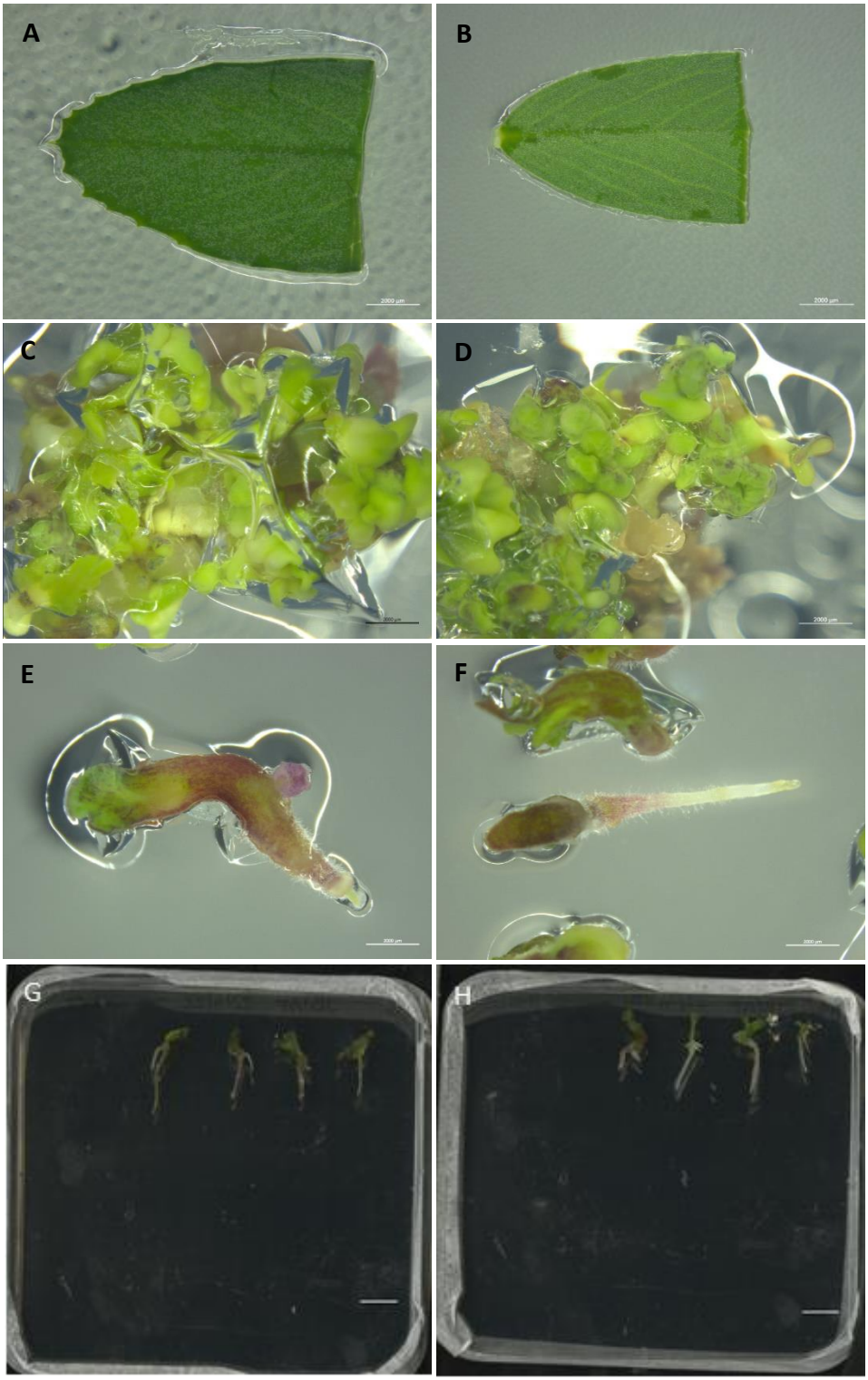

Figure 4 *- Development stages of* ***M.*** sativa *during somatic embryogenesis.* **(A)** *M. sativa* Wild type leaf on B5H medium, **(B)** *M. sativa SIMKK RNAi* leaf on B5H medium, **(C)** *M. sativa* Wild type callus on B50 medium after 23 days, **(D)** *M. sativa SIMKK RNAi* callus on B50 medium after 23 days, **(E)** *M. sativa* Wild type embryo on MMS medium for root initiation, **(F)** *M. sativa SIMKK RNAi* embryo on MMS medium for root

initiation, **(G)** *M. sativa* Wild type plantlets on MS medium, **(H)** *M. sativa SIMKK RNAi* plantlets on MS medium; scale bar (A-F) – 2 mm, scale bar (G, H) – 1 cm.

4.2 Phenotyping of the selected lines

For comparing the phenotypic difference of the selected lines, the plants were cultivated in Fåhraeus media containing 3 different concentrations of NaCl and treated with *P. fluorescens*. The plants in the Petri dishes were scanned using scanner once per day for 10 days (Figure 5). For evaluation of the root length measurements were taken from the main root using ImageJ software. The values measured were subtracted to obtain the differences in main root growth.

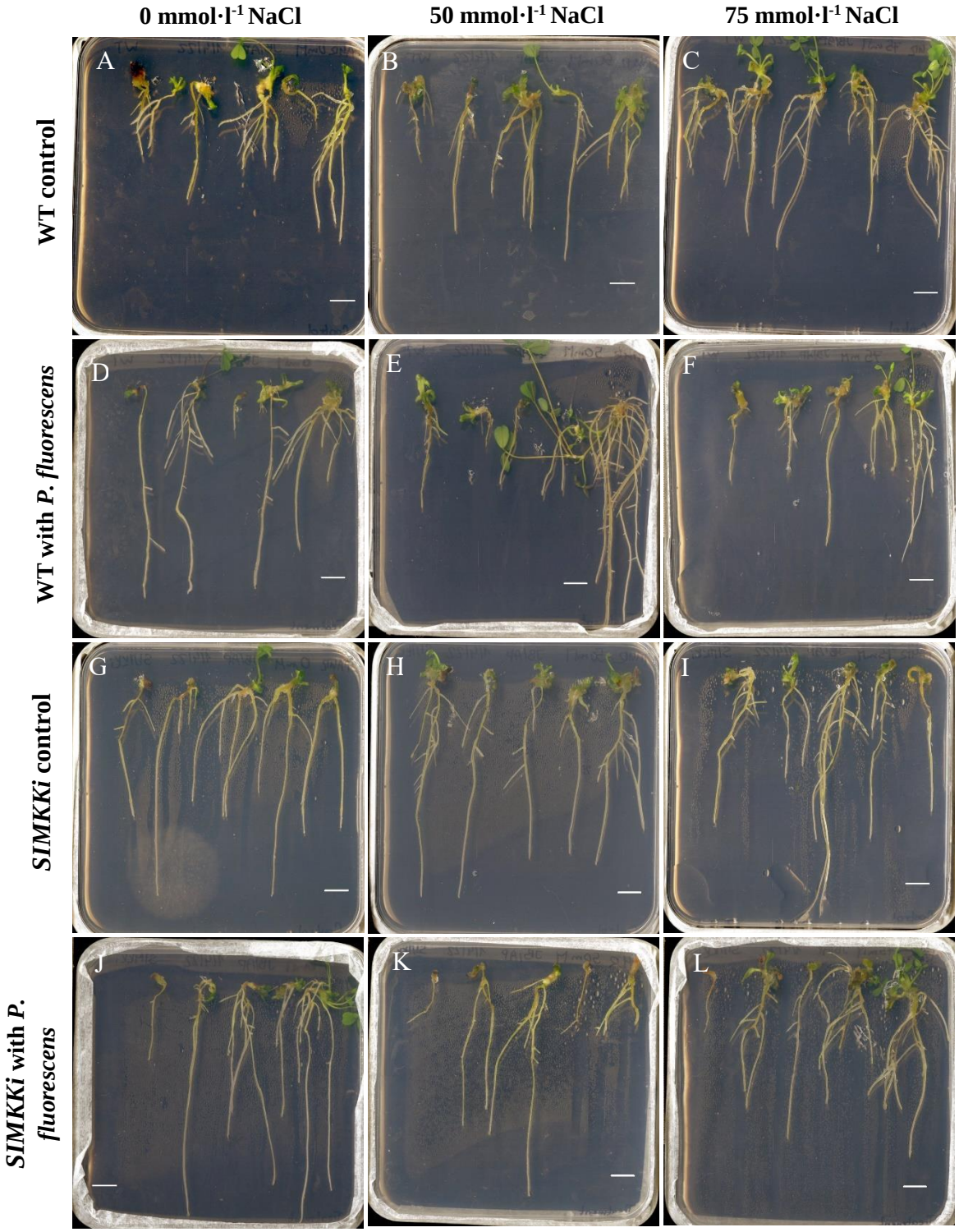
0 mmol·l-1 NaCl
50 mmol·l-1 NaCl
75 mmol·l-1 NaCl
WT control
WT with P. fluorescens
SIMKKi control
SIMKKi with P. fluorescens
A
B
C
D
E
F
G
H
I
J
K
L

Figure 5 – Comparison of *M. sativa* WT and *M. sativa SIMKK RNAi* plants under control and *P. fluorescens* co-cultivation during salt stress after 10 days. **(A)** *M. sativa* WT control at 0 mmol·l^{-1} NaCl; **(B)** *M. sativa* WT control at 50 mmol·l^{-1} NaCl; **(C)** *M. sativa* WT control at 75 mmol·l^{-1} NaCl **(D)** *M. sativa* WT with *P. fluorescens* at 0 mmol·l^{-1} NaCl; **(E)** *M. sativa* WT with *P. fluorescens* at 50 mmol·l^{-1} NaCl; **(F)** *M. sativa* WT with *P. fluorescens* at 75 mmol·l^{-1} NaCl; **(G)** *M. sativa SIMKK RNAi* control at 0 mmol·l^{-1} NaCl; **(H)** *M. sativa SIMKK RNAi* control at 50 mmol·l^{-1} NaCl; **(I)** *M. sativa SIMKK RNAi* control at 75 mmol·l^{-1} NaCl; **(J)** *M. sativa SIMKK RNAi* with *P. fluorescens* at 0 mmol·l^{-1} NaCl; **(K)** *M. sativa SIMKK RNAi* with *P. fluorescens* at 50 mmol·l^{-1} NaCl; **(L)** *M. sativa SIMKK RNAi* with *P. fluorescens* at 75 mmol·l^{-1} NaCl. Scale bar = 1 cm.

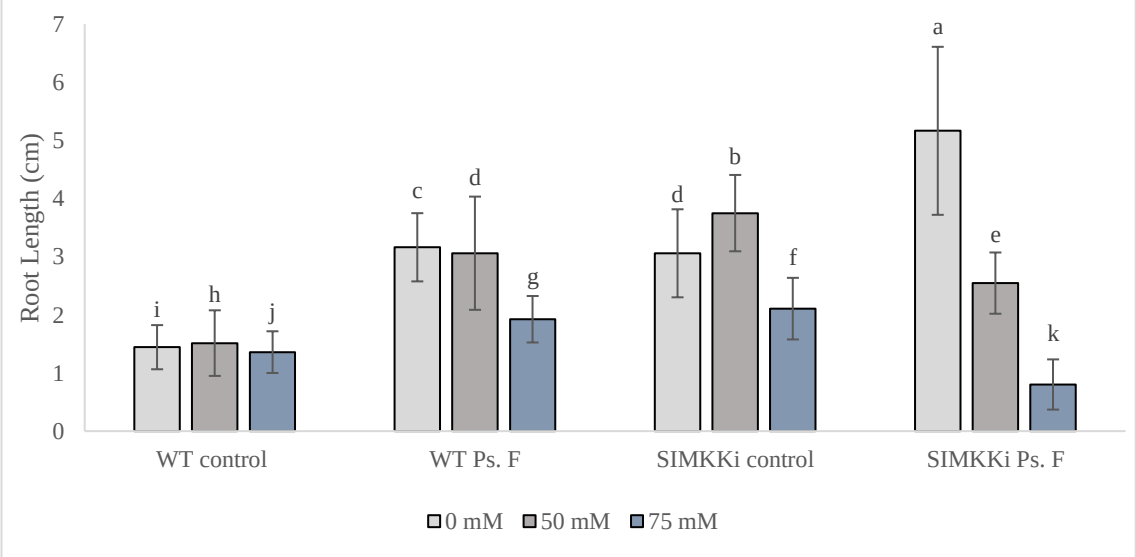

Figure 6 Graphical representation of difference in average root length in control and *P. fluorescens* treated plants of *M. sativa* Wild Type and *SIMKK RNAi* lines under salt stress after 10 days of growth. Data is represented as mean ± SD, $n = 60$; $p < 0.05$.

After 10 days of treatment, the roots of *M. sativa* wild type and SIMKK RNAi lines were compared. In the case of wild type plantlets mock treatments, the average root length were 1.4438 cm, 1.5138 cm and 1.3588 cm for 0, 50 and 75 mmol·l^{-1} NaCl respectively (Figure 5 A-C and Figure 6). However, in the case of wild type plantlets which are treated with *P. fluorescens* were having an average root length of 3.1618 cm, 3.0598 cm and 1.924 cm, 0, 50 and 75 mmol·l^{-1} NaCl respectively (Figure 5 D-F and Figure 6). Here in the case of 0 and 50 mmol·l^{-1} NaCl, there is a 100% increased in the mean root length. In *M. sativa SIMKK RNAi* mock treated plantlets, the overall length

were 3.0582 cm, 3.7492 cm and 2.1064 cm for 0, 50 and 75 mmol·l^{-1} NaCl respectively which was twice the length compared to WT mock treated plants (Figure 5 G-I and Figure 6). Mean values for *SIMKK RNAi P.fluorescens* treatment were 5.1646 cm, 2.5456 cm and 0.802 cm which showed increase in the root length for 0 mmol·l^{-1} but had a significant decrease of 50% in 75 mmol·l^{-1}. With *P. fluorescens* treatment, the growth enhancement was highest in both WT and *SIMKK RNAi* plantlets under no salt stress (Figure 5 J-L and Figure 6). There is a trend of growth retardation with 75 mmol·l^{-1} NaCl concentration in both lines. How-ever with bacterial co cultivation, there was a slight increase in the root length of WT plants but in case of *SIMKK RNAi* the root length was reduced by 50%.

Salt stress has negative impact on root growth by causing disbalance in plant growth by damaging the cells and disrupting osmotic balance in cells (Mahajan & Tuteja, 2005; Horie *et al*., 2011). Report suggests the involvement of microbial production of indole-3-acetic acid (IAA) which is a phytohormone belonging to the auxin family, which has the impact on root growth by cell elongation, initiating of roots or cell division as well even under stress (Phillips *et al*., 2011). IAA is a commonly produced hormone by plant growth promoting rhizobacteria (PGPR) like *P. fluorescens, Enterobacter, Rhizobium, Azotobacter, Azospirillum* etc. (Apine & Jadhav, 2011; Meliani *et al*., 2017). It was discovered that the IAA producing *Bacillus velezensis* FMH2 increased plant root length and lateral root production that improved tomato salinity tolerance (Masmoudi *et al.,* 2021). *M. sativa SIMKK RNAi* line showed significantly high difference in root growth when compared to its control plantlets.

Liu *et al.,* (2021) reported the role of *P.azotoformans* with enhanced ACC deaminase production helped in lowering stress ethylene production in tomato plants under salinity induced stress. Another study by Ji *et al.,* (2020) showed the effect of ACC deaminase produced by halotolerant *Glutamicibacter* sp. YD01 reduced stress ethylene production in rice seedlings when grown under stress caused by 200 mmol·l^{-1} NaCl. ACC deaminase enzyme produced by *Streptomyces* sp. GMKU 336 was capable of down-regulating the ACC oxidase (ACO1) and the ethylene responsive element binding protein (EREBP1) genes in rice plants when grown in salt concentration of 150 mmol·l^{-1} NaCl (Jaemsaeng *et al.,* 2018). In the present study, both wild type and *SIMKK RNAi* mock treated groups, the highest root growth was measured under 50 mmol·l^{-1}.

4.3 Visualization of *P. fluorescens* colonization in the selected plants roots

Root colonisation was observed using Axio Zoom Microscope (Carl Zeiss, Germany) with an excitation wavelength of 488 nm and emission wavelength of 509 nm using both brightfield and GFP channels. The final pictures were generated by merging the channels to obtain the images shown in Figure 7.

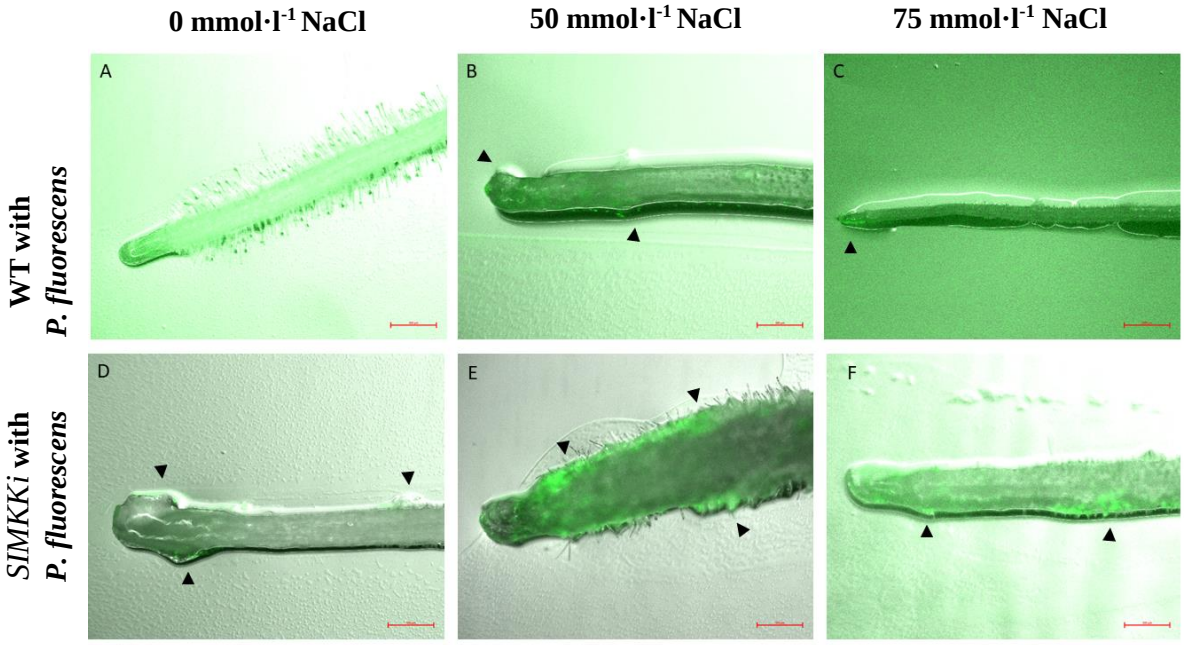

Figure 7 Zoom Microscopic images of *M. sativa* Wild Type and *SIMKK RNAi* lines with GFP tagged *P. fluorescens* under salt stress. Arrows showing colonised *P. fluorescens*. **(A)** *M. sativa* WT with *P. fluorescens* at 0 mmol·l^{-1} NaCl; **(B)** *M. sativa* WT with *P. fluorescens* at 50 mmol·l^{-1} NaCl; **(C)** *M. sativa* WT with *P. fluorescens* at 75 mmol·l^{-1} NaCl; **(D)** *M. sativa SIMKK RNAi* with *P. fluorescens* at 0 mmol·l^{-1} NaCl; **(E)** *M. sativa SIMKK RNAi* with *P. fluorescens* at 50 mmol·l^{-1} NaCl; **(F)** *M. sativa SIMKK RNAi* with *P. fluorescens* at 75 mmol·l^{-1} NaCl. Scale bar – 500 µm.

The microscopic observation of the proved that the root colonization was strongest in *M. sativa SIMKK RNAi* line under 50 and 75 mmol·l^{-1} NaCl concentrations (Figure 7 E & F) but notably the growth was found to be reduced in both these cases (Figure 7). There is a significantly lower colonization that can be observed in *M. sativa SIMKK RNAi* line under 0 mmol·l^{-1} but had a significant growth enhancement compared to mock treated plants (Figure 6 D & Figure 5). In the case of *M. sativa* WT plants, there was significantly

low amount of colonisation observable in the microscopic images but had higher root growth when compared to its mock treated plantlets (Figure 6 A-C & Figure 5).

4.4 Viability determination by propidium iodide using Spinning Disk Microscopy

The viability of the roots was determined by propidium iodide staining using Spinning disk microscopy. In *M. sativa* WT mock treated roots, the number of dead cells were mostly similar in all the three concentration of salt (Figure 8). This was comparable with the average root length observable from the figure 5. But in 50 mmol·l^{-1} mock treated *M. sativa* WT plant root, the cell morphology was found to be different from that of 0 mmol·l^{1} and 75 mmol·l^{-1} NaCl (Figure 8 A-C). In the case of *M. sativa SIMKK RNAi* mock treated plants, the cells seem to agree with root length (Figure 8 G-I and Figure 7). The cell death seems to be lower in 0 mmol·l^{-1}, slightly increased in 50 mmol·l^{-1} and highest in 75 mmol·l^{-1}. The cell morphology was also different in 50 mmol·l^{-1} and 75 mmol·l^{-1} concentrations when compared to its respective controls (Figure 8 G-I). The *P. fluorescens* treatment in the *M. sativa* WT plants doesn't seems to have much slightly positive influence in the cell viability and cell morphology of plant roots with 0 and 50 mmol·l^{-1} NaCl concentrations, but in 75 mmol·l^{-1}, the cell death was observed to be higher (Figure 8 D-F). This was also in agreement with the root length which showed almost 100% increase in the length in 0 and 50 mmol·l^{-1} concentration (Figure 6). In the case of *M. sativa* WT plants with *P. fluorescens* on 75 mmol·l^{-1} NaCl, higher cell death was observed which could be the reason of reduced root length when compared to the 0 and 50 mmol·l^{-1} treated plants. In *M. sativa SIMKK RNAi* plants with *P. fluorescens* treatment under 0 mmol·l^{-1} NaCl had the highest viability and root length. This samples were also showing much less bacterial biofilm but had significantly higher growth when compared to its other respective treatment groups (Figure 7 J and Figure 5). In the case of *M. sativa SIMKK RNAi* plants with *P. fluorescens* in 50 and 75 mmol·l^{-1} NaCl, they showed higher cell death and the cell morphology was different when compared to its respective control (Figure 7 K-L). The bacterial colonisation was also found to be significantly increasing with increasing concentration of NaCl, how ever was inversely proportional to the root length (Figure 7 K-L and Figure 5).

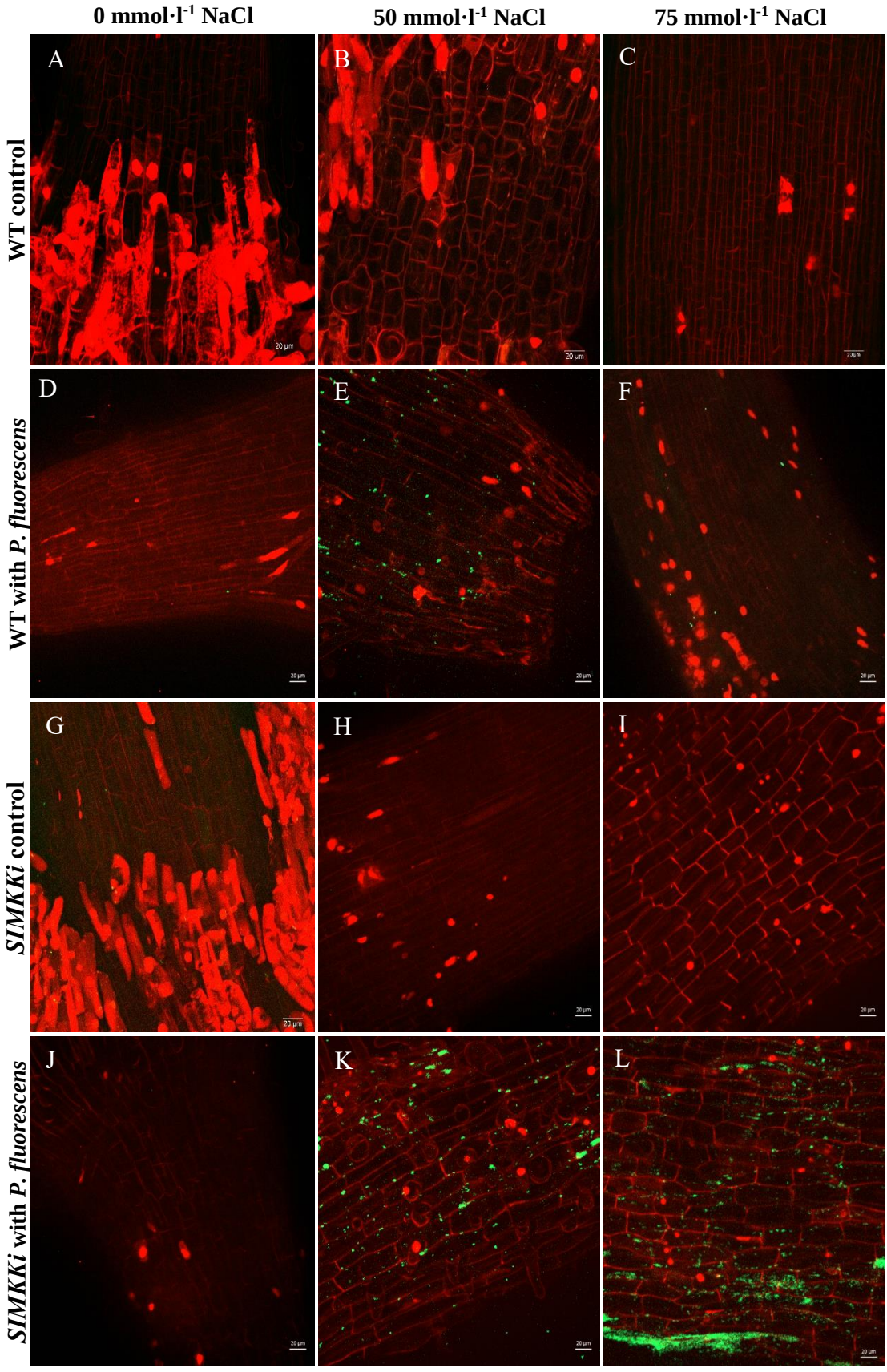
0 mmol·l-1 NaCl
50 mmol·l-1 NaCl
75 mmol·l-1 NaCl
WT control
WT with P. fluorescens
SIMKKi control
SIMKKi with P. fluorescens
A
B
C
D
E
F
G
H
I
J
K
L

Figure 8 – Live dead cell imaging of *M. sativa* WT and *SIMKK RNAi* plants under control and *P. fluorescens* co-cultivation during salt stress after 10 days. **(A)** *M. sativa* WT control at 0 mmol·l^{-1} NaCl; **(B)** *M. sativa* WT control at 50 mmol·l^{-1} NaCl; **(C)** *M. sativa* WT control at 75 mmol·l^{-1} NaCl **(D)** *M. sativa* WT with *P. fluorescens* at 0 mmol·l^{-1} NaCl; **(E)** *M. sativa* WT with *P. fluorescens* at 50 mmol·l^{-1} NaCl; **(F)** *M. sativa* WT with *P. fluorescens* at 75 mmol·l^{-1} NaCl; **(G)** *M. sativa SIMKK RNAi* control at 0 mmol·l^{-1} NaCl; **(H)** *M. sativa SIMKK RNAi* control at 50 mmol·l^{-1} NaCl; **(I)** *M. sativa SIMKK RNAi* control at 7522 mmol·l^{-1} NaCl; **(J)** *M. sativa SIMKK RNAi* with *P. fluorescens* at 0 mmol·l^{-1} NaCl; **(K)** *M. sativa SIMKK RNAi* with *P. fluorescens* at 50 mmol·l^{-1} NaCl; **(L)** *M. sativa SIMKK RNAi* with *P. fluorescens* at 75 mmol·l^{-1} NaCl. Scale bar = 20 µm.

5. CONCLUSION

In the current work, the main focus was to evaluate the effect of salt stress mitigation property of PGP bacterium *P. fluorescens* strain R562 (GPF-tagged) on two lines of *M. sativa* (Regen-SY wild type and Regen-SY *SIMKK RNAi*). Initial part of the thesis was focused on the describing the importance of *M. sativa*, its well-known ability of symbiosis with nitrogen fixing bacteria. In this part we tried also the describe the role of MPKs in stress managements and associated properties. For the cultivation of plants somatic embryogenesis was adopted from surface sterilised leaves collected from the selected lines. The plants with similar size and age were selected for all the experiments. Phenotyping of the lines in the influence of NaCl induced stress confirmed that *M. sativa* WT plants have similar root length but *M. sativa SIMKK RNAi* lines were having slight growth retardation when compared to its respective controls on all the three different concentrations of NaCl used (0, 50, 75 $mmol \cdot l^{-1}$). How ever when the plants were co cultivated with bacteria, they have significantly higher root length compared to their mock treatments. After phenotyping all the plants co-cultivated with bacteria was subjected to colonization using fluorescence microscopic analysis which proved all the treatment have different levels of colonisation patterns. It was quite surprising to see that the highest number of colonies were found in *M. sativa SIMKK RNAi* lines under 50 and 75 $mmol \cdot l^{-1}$ NaCl and it had an inverse correlation with the root length compared to the respective mock treated *SIMKK RNAi* plants as well as treated WT plants. Trend in cell viability was also supporting the results from the phenotyping as well as colonisation. It was also possible to connect the cell morphology in 50 $mmol \cdot l^{-1}$ and 75 $mmol \cdot l^{-1}$ NaCl in *M. sativa SIMKK RNAi* with *P. fluorescens* treatment to colonisation and root length since they had different morphology when compared to the mock controls. It will be quite interesting to perform more phenotyping analysis as well as biochemical analysis to check the levels of different analogs of MAPKs, the role of cytoskeletal structures etc., which could be a future perspective of this work.

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LIST OF SYMBOLS AND ABBREVIATIONS

ACC	1-aminocyclopropane-1-carboxylate deaminase
ACO1	1-aminocyclopropane-1-carboxylate deaminase oxidase
B5-0	medium for embryo formation
B5-H	callus inducing medium
cv.	cultivar
EREBP1	ethylene responsive element binding protein
FAO	Food and Agriculture Organization of the United Nations
FDA	fluorescein diacetate
GFP	green fluorescent protein
IAA	indole-3-acetic acid
L.	Linnaeus, Carl (Carl von Linné)
LB	Luria-Bertani medium
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
$mE \cdot m^{-2} \cdot s^{-1}$	SI unit of photon flux density
MMS	medium for embryo germination
MS	Murashige & Skoog medium
NA	numerical aperture
nm	nanometers
OD600	optical density at 600 nm
PGPB	plant growth promoting bacteria
PGPR	plant growth promoting bacteria
PI	propidium iodide
Ps.	*Pseudomonas*
RNAi	RNA interference
RPM	revolutions per minute
SD	spinning disk
SIMK	salt-stress induced mitogen-activated kinase
SIMKK	salt-stress induced mitogen-activated kinase kinase

v/v	volume to volume
WT	wild type