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Identification of fungi that cause tip rot of carrot and determine effect of storage temperature and cultivar on tip rot development

Rizan Amin Manla Mohamad

Master in plant science

Abstract:

Carrot is one of the important vegetables in Norway, and after harvesting it stored in cold storage for up to 7 months. During such long storage time, there is great yield and quality losses due to various diseases, and one of the most severe problem is tip rot of carrots. Tip rot starts as discoloration and necrosis from the tip (base) of the taproot and goes upwards in the edible part of the root. The symptom appears during and after cold storage but may be seen already at harvesting. In this study, we hypothesized that fungi have a role in tip rot disease development. To identify the primary causal agents of tip rot in Norway, we took samples from four fixed fields from Rogaland, Trøndelag, Innlandet, Viken and six commercial cold storages, in Rogaland, Trøndelag, Innlandet and Viken. Selection of fields and cold storages and sampling were caried out in close collaboration with advisors in Norsk Landbruksrådgiving (NLR). Based on morphological (symptoms appearance, isolation, and microscopy) and molecular approaches like (DNA sequencing and metabarcoding), the most frequently appearing pathogens were selected for further studies. The pathogenicity of the candidate pathogens was tested on four carrot cultivars, Dailyance, Brillyance, Namdal and Romance. In addition, the effect of temperature on the latent period of tip rot was determined by incubating inoculated carrots with candidate pathogens at 0+1°C, 3±1°C and 6±1°C. Several fungi were found on carrots with tip rot symptom, but the most abundant were *Mycocentrospora acerina*, *Cylindrocarpon* spp., *Fusarium* spp. and *Dictyostelium* spp. Those were used for pathogenicity test. The latent period was significantly different among storage temperature for all candidates ($P < 0.05$), and the latent period become shorter with an increase in temperature. There was variation in disease development between pathogens i.e. the development of *M. acerina* was faster than *Cylindrocarpon* spp., and *Fusarium* spp., while the lowest disease development was for *Dictyostelium* spp. Disease severity (lesion size) expressed as Area under disease progress (AUDPC) was significantly different among storage temperature for all candidates and between the pathogens i.e. *M. acerina* had the highest severity (AUDPC) while *Dictyostelium* spp., had the lowest ones. All the tested pathogens showed both the wet and dry type tip rot. The tested pathogens showed a slightly difference symptoms (discoloration) on carrot tissue when observed on the outside surface of the carrot and inside after splitting the carrot. The most typical symptom of *M. acerina* was brown to black discoloration starts from the tip of taproot, and the infection goes inside the carrots (xylem tissue) of the carrot with wet type black discoloration. On advanced infection, chlamydospores formed on the surface of the carrot tissue and gave the characteristic black symptom of liquorice rot. The symptom for *Cylindrocarpon* spp. was brown or dark brown to black infection starts from the tip of taproot, and the infection inside the carrot was as drying the whole area (core with surrounded flesh) and getting brown to black. The symptom of *Fusarium* spp. was light brown, brown and sometimes pink, while the infection inside the carrot was as dry, and brown core goes deep in the cylindrical core of the carrot tissue. *Fusarium* infection is mostly dry type, but it could be wet too. The symptom of *Dictyostelium* spp. was light brown, brown to dark brown, and the infection inside the carrot was as dry

and brown core goes deep in the core. In conclusion, tip rot of carrots is a disease complex of stored carrots caused by different fungi with possible interaction with other microorganisms and physiology of the carrot taproot. Tip rot of carrot is caused by biotic agents. Tip rot causal agent identification based on symptom on the tip of the carrot is difficult, so one has to split the carrot and see how deep the symptom goes in the taproot. Even the splitting and symptom was not sufficient to correctly identify the causal agent, so one has to confirm the pathogen structure as spores, chlamydospores and hyphal structure under a microscope or conduct DNA based identification.

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Rizan Amin Manla Mohamad May 2021

Contents

Abbreviations:

PDA: Potato Dextrose Agar.

PGA: Potato Carrot Agar.

RH-19: Samples at harvesting time from fixed field Rogaland in 2019.

R.H.20.TR: Samples at harvesting time from fixed field Rogaland in 2020 with tip rot.

T. H20.TR: Samples at harvesting time from fixed field Trøndelag in 2020 with tip rot.

V.H.20: Samples at harvesting time from fixed field Viken in 2020.

V.H.20. S.5P: Samples from location 5 Sem at the fixed field in Viken in 2020.

L1.20: Samples after storage time from commercial storage Viken in 2020.

L3.20: Samples after storage time from commercial storage Rogaland in 2020.

L4.20: Samples after storage time from commercial storage Rogaland in 2020.

L5.20: Samples after storage time from commercial storage Innlandet in 2020.

L6.20: Samples after storage time from commercial storage Innlandet in 2020.

L7.20: Samples after storage time from commercial storage Viken in 2020.

L2.20: Samples after storage time from fixed field Viken in 2020.

RS-19: Samples after storage time from fixed field Rogaland in 2019.

TS-19: Samples after storage time from fixed field Trøndelag in 2019.

V.L-19: Samples after storage time from fixed field Viken in 2019.

RG-19: Samples at growing season (mid-season) from fixed field Rogaland in 2019.

TG-19: Samples at growing season (mid-season) from fixed field Trøndelag in 2019.

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1.Introduction:

1.1. The carrot plant:

Cultivated carrot (*Daucus carota*. L) belongs to the botanical family Apiaceae, which was known before as the Umbelliferae family (Rubatzky et al., 1999). Apiaceae is a large family, and it involves 466 genera with about 3820 species (Spooner, 2019). Apiaceae contains plants that are annual, biennial or perennial, and besides carrot it includes many vegetable crops. Plants in the family have different beneficial and edible parts such as roots, tubers, stems, petioles, leaves, flowers, fruits, and seeds. Popular and important crops from the family include carrot, celery, parsnip, and cilantro (Rubatzky et al., 1999). Carrot is especially important because of its high content of vitamin A, carbohydrates, and carotene (α, β), and the consumption of it increases every year in many countries.

Carrot plants consist of the foliage part over the ground and a root underground, the root is the primary organ of agricultural importance. The outer layer of the root is the skin (Epidermis), and the innermost part call flesh or phloem while the central part call core or xylem, the point of connection between the foliage and the root call collar and the rest part is storage root which end up by taproot and it includes many secondary roots. The area between the taproot and the storage root is base (tip) (Rubatzky et al.,1999) (Fig. 1).

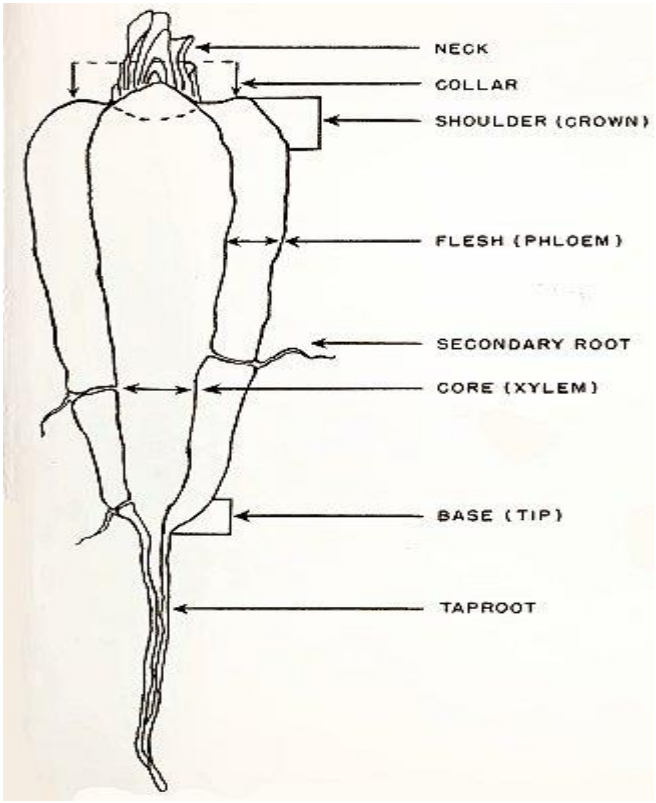

Fig.1. Different parts of carrot root. Available at: http://www.carrotmuseum.co.uk/carrotroot.html.

1.2. Carrots in Norway:

There is a big increase in carrot and turnip production worldwide, about 10 million tonnes since 2010 (FAO, 2019).

In Norway, carrots have been planted since the 1600s, and at that time it was violet in colour; however, orange, or red carrots were mentioned already in 1471 (Balvoll, 1999). Carrots are an important vegetable in Norwegian diet, and the increase in consumption has led to a yearly increase in the cultivated area in last twenty years (Statistics Norway, 2019). The sowing time is from early spring (March) until the end of May, and harvesting is from July until late autumn, before the frost sets in. After harvest, carrots may be stored in cold rooms for 5 to 7 months, i.e., until April-May (Franke, 2013). The best suitable storage conditions for carrots are 0°C and 98-100% relative humidity (Balvoll, 1999). However, during the storage period, the carrots are vulnerable for many diseases, and carrot losses in store may vary between 10 to 40 % (Franke, 2013). In addition, small and not matured carrots dry faster than larger and matured carrots, and the small ones may be more susceptible for infection of *Botrytis cinerea* (grey mould) in the storage (Balvoll, 1999).

1.3. Storage diseases of carrots:

There is variation in the ability of the fungal pathogens to infect carrots. Some can penetrate the plant tissue directly whereas others need wounds or natural openings to enter the root, and fungi can infect the plants while the symptoms show up later (latent infections) (Korsten and Wehner, 2003; Rubatzky et al., 1999). Many fungi can attack carrots in the field, at time of harvest or after storage. Most fungal pathogens survive in the soil or on plant residues because many of them form resting structures like chlamydospores. Therefore, the primary source of contamination is found in the soil (Korsten and Wehner, 2003). During the storage period, the carrots are vulnerable for many fungal diseases, and in Norway, the most problematic and vital carrot storage pathogens are *Botrytis cinerea*, *Fibularhizoctonia carotae*, *Mycocentrospora acerina*, *Sclerotinia sclerotiorum* (Hermansen et al., 2012). Some of the important carrot storage pathogens are:

1.3.1-*Mycocentrospora acerina* (Liquorice rot):

Mycocentrospora acerina (R. Hartig) Deighton (synonym: *Centrospora acerina* (R. Hartig) A.G. Newhall), was first studied by Hartig in 1880 (Evenhuis, 1998). This pathogen causes a disease on carrots called liquorice rot. The fungal classification is: Fungi, Ascomycota, Pezizomycotina, Dothideomycetes, Pleosporomycetidae, Pleosporales, *Mycosentrospora* (NBN atlas, 2021).

1.3.1.1- Epidemiology:

Mycocentrospora acerina is a necrotrophic pathogen and mostly a wound pathogen, and the disease can develop at cold store temperatures (Davies et al., 1981; Louarn et al., 2012). In addition, the infection can appear on the leaves in the field, but it does not cause a severe reduction in the yield

(Hermansen, 2008). *Mycocentrospora acerina* has about 90 host plants, including weeds *(*e.g., *Viola arvensis*), ornamentals (e.g., *Viola tricolor*), and vegetables like carrot, parsley, and celery (Hermansen, 1992). The fungus is soil borne, and it stays in the soil without its hosts as conidia (asexual spores) or chlamydospores (resting spores), and the latter can survive in soil for up to eight years (Hermansen, 2008). When there is a host near or in contact with the spores, they will germinate, and the mycelium or hyphae infect the carrots through wounds or weak cells of the plant tissue (Hermansen et al., 2012; Rubatzky et al., 1999). The optimal temperature for fungal growth is 18°C even though the disease can develop at -3°C (Hermansen, 2008). The infection risk increases with increasing amount of inoculum in the soil, soil humidity, and injury of the carrots (Rubatzky et al., 1999). No sexual phase of the fungus is known (personal communication B. Asalf, Norwegian Institute of Bioeconomy Research).

1.3.1.2-Symptoms of liquorice rot:

The fungus can go deep into the carrot, and it forms a blackish, porous, and juicy rot, while in newly infected tissue, the colour of the decay is light brown (Fig. 2) (Hermansen et al., 2012). In addition, symptoms can appear on the leaves as irregular brownish to blackish spots along the edges (Hermansen, 1992).

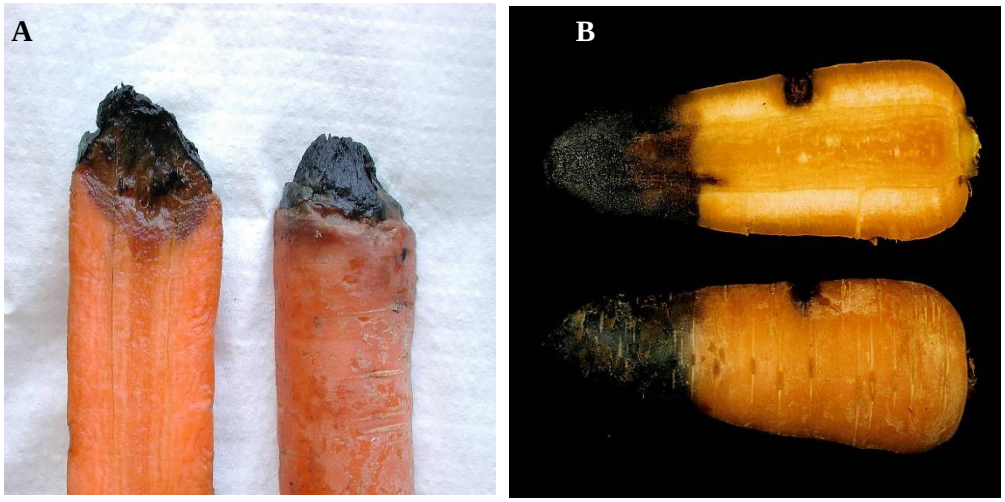

Fig.2. *Mycocentrospora acerina* symptoms on carrots; (A) photo by (E. Fløistad, NIBIO); (B) photo by (A. Hermansen NIBIO). Available at: https://www.plantevernleksikonet.no/l/oppslag/1270/.

1.3.2-*Cylindrocarpon* spp./*Ilyonectria* spp. (Cylindrocarpon root rot):

The taxonomy of *Cylindrocarpon* sp. is: Fungi, Ascomycota, Pezizomycotina, Sordariomycetes, Hypocreomycetidae, Hypocreales, Nectriaceae, *Ilyonecteria* (Mycobank 2021). Based on the hosts, the genus can be divided in two groups (Sweetingham, 1983): 1. Optional plant parasites with woody plants as favourable hosts. 2. Soil borne, which specially attack plant roots, producing chlamydospores as a resting spore, and the sexual stage call *Ilyonecteria.* Sometimes the pathogen is called *Cylindrocarpon destructans* and sometimes *Cylindrocarpon* spp. because there maybe more than one species.

1.3.2.1. Epidemiology:

Cylindrocarpon spp. are soilborne fungi, and they cause root rot diseases in many hosts, such as ginseng, carrots, conifer, and fruit trees (Seifert et al., 2003). It produces chlamydospores as a resting spore, so it can survive in the soil for long time under harsh conditions (Taylor, 1964). The chlamydospores germinate under suitable conditions, e.g., warm, and moist weather but they can germinate also in cold storage (Dumroese and James, 2005). Germinated spores infect injured roots in the soil if they contact the roots in the field or in storage (Korsten and Wehner, 2003; Ziezold et al., 1998). If injuries occur at harvest and the carrots are moved to the storage before washing them, the carrots may become infected because of chlamydospores remaining in the soil (Korsten and Wehner, 2003; Ziezold et al., 1998). Water from rain or irrigation can spread micro and macro conidia of *C. destructans* among carrot plants; the fungus can survive in plant debris (Dumroese and James, 2005).

1.3.2.2. Symptoms of *Cylindrocarpon* spp. (root rot) in storage:

There is very limited published literature regarding symptoms of *Cylindrocarpon* spp. on carrots in storage. Nærstad (2015) in Norway described a discoloration of infected tissue as dry and dark brownish.

1.3.3. *Fibularhizoctonia carotae* (Carter rot):

Fibularhizoctonia carotae (Rader) G.C. Adams & Kropp (teleomorph *Athelia arachnoidea*) led to storage losses of 50 to 70% in stored carrots in Denmark (Rader, 1948). It may also be a pathogenic on cabbage, turnips, beets, and celeriac in Norway (Hermansen, 2008). The taxonomy of *F. carotae* is: Fungi, Basidiomycota, Agaricomycotina, Agaricomycetes, Atheliales, Atheliaceae, *Rhizoctonia/Athelia* (Mycobank 2021).

1.3.3.1. Epidemiology:

The fungus survives in the soil as sclerotia (a resting structure in a compact mass of hardened and melanised fungal mycelium) or as mycelium (Hermansen, 2008), *Fibularhizoctonia* is the asexual stage and the sexual stage is *Athelia* sp. which occurs on the leaves (Adams and Kropp, 1996). The infection of the carrots occurs in the field, and the symptoms appear later in storage (Rader, 1948), or the carrots can get infected from fungal structures following soil or plant debris transported with carrots to the storage. In addition, infested pallet boxes can be a source of inoculum and infect the carrots (Årsvoll, 1971). The fungus can develop and grow at temperatures as low as -3°C (Hermansen, 2008).

1.3.3.2. Symptoms of the carter rot disease in the storage:

The typical symptoms of the fungus according to Rader (1948) are: Dry and sunken lesions with whitish cottony mycelium, and small white hyphal knots on the root surface (Fig. 3). On the sunken spots, whitish to yellowish hyphae will form, and the spot sizes can differ under different environmental conditions and different cultivars. Sometimes the fungus grows up in the root and forms a light brown

and soft rot, and in storage the dispersal of the pathogen from diseased to healthy carrots is by fungal mycelium (Hermansen, 2008; Rader, 1948). High relative humidity and the existence of moisture on the carrot surface can make the development of the fungus faster (Hermansen, 2008; Rader, 1948).

Fig.3. *Fibularhizoctonia carotae* symptoms on carrots. photo by (E. Fløistad, NIBIO). Available at: https://www.plantevernleksikonet.no/l/oppslag/481/.

1.3.4. *Botrytis* spp. (Grey mould):

Botrytis spp. are airborne, necrotrophic pathogen, and it can attack hundreds of crop hosts (Staats et al., 2005; Williamson et al., 2007), and among those hosts is carrot, and the fungus can infect the leaves during the growing season and infect the carrot roots in storage (Hermansen et al., 2012). The fungal taxonomy is: Fungi, Accomycota, Pezizomycotina, Leotiomycetes, Helotiales, Sclerotiniaceae, *Botryotinia* (NBN atlas, 2021). *Botryotinia* is the perfect stage, which is very rare, while *Botrytis* is the anamorph.

1.3.4.1. Epidemiology:

Botrytis spp. can exist as mycelium, micro- and macro conidia, chlamydospores and sclerotia. They release macroconidia in dry air, and sclerotia can survive under harsh conditions for several years (Holz et al., 2007). In the field and under high humidity and mild temperature the fungus easily sporulates, and it may infect the old and injured tissues at the base of petioles or the root crown or contaminate them before the storage. The dispersal of the fungus occurs by wind or water splash, and the fungus usually does not infect carrots which are in good condition. High humidity in the field or storage is crucial for infections to occur by airborne spores. The optimal temperature for fungal growth is between 23°C and 25°C; however, the infection can develop in storage at 0°C (Hermansen, 2008; Lockhart et al.,1974).

1.3.4.2. Symptoms of the grey mould on carrots:

Botrytis spp. can grow at low temperatures (above freezing degree) and the symptoms depend on the plant part that is attacked (Elad et al., 2004). Typical symptoms are water-soaked tissues with a spongy

appearance, covered by mould (mycelium and conidial spores), and sometimes the symptoms look like dark brownish or blackish lesions (Lockhart et al., 1974). In cold store a white fungal mycelium covers the carrot and inside this cover it forms sclerotia, which are greyish white in the beginning and eventually become blackish (Fig. 4) (Hermansen et al., 2012).

Fig.4. *Botrytis cinerea* symptoms on carrots; photo by (E. Fløistad NIBIO).

1.3.5. *Alternaria radicinia* (Black root):

Alternaria radicina Meier, Drechsler & E.D. Eddy or syn. *Stemphylium radicinum* (Meier, Drechsler & E.D. Eddy) Neergaard (NBN atlas 2021). It can attack carrot and sometimes other vegetable crops like parsley and celery, and it can infect both the foliage and the roots (Rubatzky et al., 1999). It causes a black root disease on carrots, and it is a saprophytic pathogen and can live in dead organic matter in the soil or on dead carrot tissue (Lauritzen, 1926). The pathogen can infect uninjured carrots, but the most favourable tissue for the pathogen is injured, small (older or younger) wounds on the carrot skin (Lauritzen, 1926). *Alternaria radicina* does not have a known sexual stage and may therefore be classified under Deuteromycetes; however, its taxonomy is commonly classified as: Fungi, Ascomycota, Pezizomycotina, Dothideomycetes, Pleosporales, Pleosporaceae, *Alternaria* (NBN atlas, 2021).

1.3.5.1. Epidemiology:

Alternaria radicina is a seed-borne and soil-borne pathogen, and it survives in the soil as free spores and on the plant debris as conidia (Pryor et al., 1998). The primary inoculum source is conidia in the soil and infected foliage, and the infection may occur in direct connection between roots and spores in the soil before harvest or when infected foliage contacts the roots (Rubatzky et al., 1999). High temperature and humidity stimulate spore formation in the infected lesions, and wind helps in spore dispersal in the field (Rubatzky et al., 1999). Carrots in storage are contaminated during harvest, or the soil on

the carrots contain conidia that may infect during cold store (Maude, 1966). If humidity is high, the pathogen can infect the carrots at a temperature as low as 0.6°C (Maude, 1966).

1.3.5.2. The symptoms of black root on carrots:

In the field, symptoms on the foliage are small necrotic spots with chlorotic margin, which can coalesce to blackish necrosis covering the whole leaf (Rubatzky et al., 1999). A black decay of the lower petioles may lead to a black ring of decay at the point where the petiole is attached to the root (Fig. 5) (Naqvi, 2004). Symptoms on roots in storage are dry, black sunken lesions on the surface of carrot taproots (Naqvi, 2004).

Fig. 5. Black rot symptoms on carrots; (Pryor, 1993). Available at: Pryor, B. M. 1993. The occurrence of *Alternaria radicina* on carrot seed and in soil. M.S. thesis. University of California, Davis.

1.3.6. *Pythium* spp. (Cavity spot):

Cavity spot is found worldwide in carrots, and it may lead to severe yield reduction. The causal agents of cavity spot on carrots are several species within *Pythium* spp. (Naqvi, 2004). In Norway, five *Pythium* spp. have been isolated from symptomatic carrots, including *P. intermedium*, *P. sulcatum*, *P. sylvativum*, *P. viola* and *P. vipa* (Hermansen et al., 2007). The taxonomy of the pathogen is: Chromista, Oomycota, Peronosporea, Peronosporales, Pythiaceae, *Pythium* (NBN atlas, 2021).

1.3.6.1. Epidemiology:

Cavity spot is caused by terrestrial *Pythium* spp., with many different hosts (Hermansen et al., 2007). The pathogen survives in the soil as resting spores (oospores), and some species of *Pythium* spp. can survive as saprophytes on dead organic matters (Hermansen, 2008). In the soil, exudates from the carrots will stimulate the resting spores to germinate, and zoospores formed in sporangia appearing on the oospores or hyphae may infect unwounded surfaces of the carrots (Vivoda et al., 1991). Some specific conditions make the carrots more susceptible for infection, such as high soil humidity, dense soil structure, and over-fertilization, and the optimal soil temperature for infection is around 15°C

(Hermansen, 2008; Hermansen et al., 2007; Vivoda et al., 1991). Cavity spot is not considered a storage disease, but in storage secondary, weak pathogens may go through the lesions caused by *Pythium* on the infected carrots and cause root rot and reduced carrot quality (Hermansen et al., 2007).

1.3.6.2. The symptoms cavity spot on carrots:

Cavity spot lesions take different shapes. However, the disease usually appears as circular, or oblong holes, but the lesions are always slightly sunken and may crack in advanced stages (Fig. 6) (Hermansen, 2008).

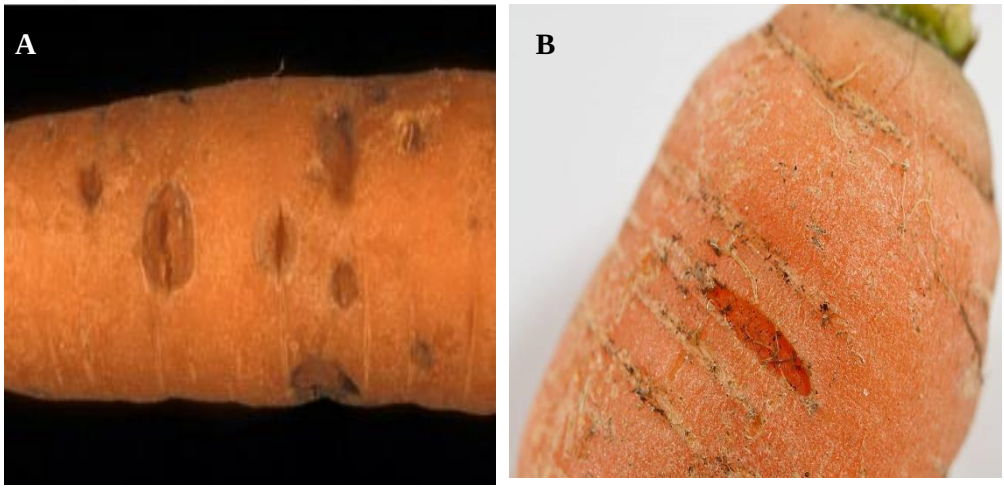

Fig.6. *Pythium* spp. Symptoms on carrots; (A) photo by (A. Hermansen, NIBIO); (B) photo by (E. Fløistad NIBIO). Available at: https://www.plantevernleksikonet.no/l/oppslag/1289/.

1.3.7. *Fusarium* spp. (Dry rot):

Dry rot is an important postharvest disease worldwide, and it causes a big reduction in yield, for example on potato and carrots. In cold store of carrot in Serbia, losses were around 20% (Stanković et al., 2015). There are different *Fusarium* spp. that can cause dry rot in carrot including *F. acuminatum* Ellis & Everh, *F. avenaceum* (Fr.:Fr) Sacc, *F. equiseti* (Corda) Sacc, *F. oxysporum* Schlechtend.:Fr, *F. redolens* Wollenweb, and *F. solani* (Mart.) Sacc (Howard et al., 1996). The taxonomy of the fungus is: Fungi, Ascomoycota, Pezizomycotina, Sordariomycets, Hypocreales, Nectriaceae, *Fusarium* (NBN atlas, 2021). *Fusarium* is the name of the imperfect (anamorph), while the known telemorphs of *Fusarium* species belong to the genera *Calonectria*, *Gibberella*, and *Nectria* (Booth, 1981).

1.3.7.1. Epidemiology:

Fusarium spp. survive in the soil as chlamydospores or as mycelium on plant debris. In the field, infections occur by direct contact of carrot roots and the fugus, or the fungus enters the carrot through root tip injuries (Brown, 1950). In storage, the dispersal occurs either by contact of mycelium from infected carrots with the healthy ones or by airborne spores (Howard et al.,1996). The favourable

temperature for the pathogen is 15-20℃, but infections may occur in the storage at 6℃ (Howard et al., 1996).

1.3.7.2. Symptoms of dry rot on carrots:

Symptoms vary among *Fusarium* spp. The most common symptoms are discoloured, dry and crumbled tissue, and in addition crown rot may appear on the collar and shoulder of the carrot (Fig. 7), as well as different sizes of cankers which mummify after drying. In some cases, slight spots may appear on the taproot, or sunken lesions in different sizes containing whitish or reddish hyphae on the main root (Brown, 1950; Howard et al.,1996).

Fig.7. *Fusarium* spp. symptoms on carrots; photo by: (B. Asalf. NIBIO). Available at: https://www.plante-vernleksikonet.no/l/oppslag/1880/.

1.3.8. *Sclerotinia sclerotiorum* (Cottony rot):

The fungus *S. sclerotiorum* (Libert) de Bary (syn. Whetzelinia sclerotiorum (Libert) Korf & Dumont) cause cottony rot on carrots. It has many host plants, e.g., vegetables such as carrots, celery, and parsley. The pathogen is widely distributed, and the infection can be in the field or in the storage. However, it causes most losses in storage (Naqvi, 2004; Rubatzky et al., 1999). Some of the most important host plants for the pathogen in Norway are potatoes, beans, and peas (Hermansen et al., 2012). The taxonomy of the fungus is: Fungi, Ascomycota, Pezizomycotina, Leotiomycetes, Helotales, Sclerotiniaceae, *Sclerotinia* (NBN atlas 2021).

1.3.8.1. Epidemiology:

The fungus can survive in soil or on dead organic material in soil for several years (Naqvi, 2004). The infection starts when sclerotia germinate and form either mycelium or fruiting bodies (apothecia), which produce airborne ascospores. The mycelium can infect the healthy carrots by direct contact and that is the way the fungus disperses in storage, while the ascospores are spread by wind in the field (Hermansen

et al., 2012; Naqvi, 2004). Ascospores do not have the ability to enter unwounded, healthy roots; however, the mycelium can infect healthy carrots (Kora et al., 2003). Under cold storage the optimal temperature for the pathogen is 3°C but it can grow even at 0°C (Kora et al., 2003).

1.3.8.2. The symptoms cottony rot on carrots:

Typical symptoms are water soaked, soft lesions and a whitish cottony mycelium covering the carrot root tissue (Fig. 8A). By the time the mycelium grows up and covers the tissue, the lesions on the root become larger. Eventually the fungus forms small and white sclerotia which increase in size over time and becomes blackish (Fig. 8B) (Hermansen et al., 2012; Naqvi, 2004).

Fig. 8. *Sclerotinia sclerotiorum* symptoms on carrots; (A) photo by (A. Hermansen Planteforsk); (B) photo by: (B. Asalf, NIBIO). Available at: https://www.plantevernleksikonet.no/l/oppslag/473/.

1.3.9. *Dictyostelium* spp. (Slime mould):

Slime moulds were reported for the first time by the German mycologist O. Brefeld in 1869 (Loomis, 1982). There is hardly any literature available about *Dictyostelium* spp. as a cold storage pathogen on carrot. The pathogen taxonomy is: Protozoa, Dictyosteliomycota, Dictyosteliomycetes, Dictyosteliaceae, *Dictyostelium* (Mycobank 2021).

1.3.9.1. Epidemiology:

The lifecycle starts when the amoebae survive in the soil or on organic matter. They normally feed on bacteria. Under harsh conditions or starvation many thousands of cells aggregate chemotactically and form slug, which is a multicellular structure. This slug produces fruiting body in the face of the soil. The fruiting body is a stalk ending up in a ball-like structure which contain a huge number of spores. This structure germinates under good conditions and release amoebae (Weijer, 2004).

1.4. Other postharvest diseases:

1.4.1. *Rhexocercosporidium carotae* (syn. *Acrothecium carotae*) (Black spot):

Rhexocercosporidium carotae (Årsvoll) U. Braun, the (syn. *Acrothecium carotae*) was described by Årsvoll in 1965 (Shoemaker et al., 2002). The fungus causes black spots on carrots during cold store (Kastelein et al., 2007; Shoemaker et al., 2002). The optimal temperature for fungal growth is 18°C, but it can grow at -3°C (Shoemaker et al., 2002). The disease is especially important in Netherlands, and it causes a big problem in organic carrot production there (Kastelein et al., 2007).

1.4.2. *Rhizoctonia solani*:

Rhizoctonia solani J.G. Kühn (teleomorph *Thanatephorus cucumeris* ((A.B. Frank) Donk) causes crown and root rot in carrot. It has a wide host range, including carrots, celery and rutabaga (Hermansen, 2008). The symptoms of the disease start as small round spots of decayed tissue, and it goes deeper over time. The disease can appear as dark brownish, dry rot around the crown, and in addition it can attack the leaves and roots in mid-season and in storage (Howard et al., 1996).

1.4.3. *Geotrichum candidum* (sour rot):

Geotrichum candidum (Link) causes sour rot on roots of carrots postharvest. It is a pathogen distributed worldwide, especially in vegetables. *Geotrichum candidum* has a wide host range, including carrot, cucumber, pumpkin, and tomato (Kim et al., 2011). The main symptoms are water-soaked lesions and a whitish mycelium. Under moist conditions the infection goes deep in the root and causes a comprehensive and whitish decay (Horita and Hatta, 2016).

1.5. Postharvest losses of carrots in Norway:

In Norway, carrots stay in storage for several months before washing and packing them for the market. During this long period of storage there can be huge losses due to various storage diseases. The losses in Norway often vary between 10 and 40% (Franke, 2013; Hermansen et al., 2012), and it may reach 50-60% (Bond, 2016; Nærstad, 2015). One of the causes of this huge loss in Norwegian packing houses is tip rot, which has been reported over the recent 10 years in Norway (Nærstad, 2015). Tip rot symptoms can be observed at harvest but mostly after storage.

There are no standard symptoms of tip rot; however, in general the symptoms start as a necrosis and discoloration from the taproot, and it goes upwards into the carrot. In a pilot study (Nærstad, 2015), carrots with tip rot symptoms from six growers in three regions (two growers from each region) in Norway were examined. Many fungi were isolated from samples and the seemingly most pathogenic fungi that were isolated are as follows: *M. acerina*, *B. cinerea* isolated from soft tip rot samples and *F. avenaceum*, *I. radicicola*/*Cylindrocarpon* spp., and *Neonectria ramularia* isolated from dry tip rot samples. However, the possible involvement of these fungi in the tip rot complex, including the ability to induce the disease, was not clarified.

-Aim of this study:

Based on the available information on post-harvest diseases of carrots and the investigation by Nærstad (2015) as described above, it was hypothesized that fungi are likely to play a principal role in the tip rot disease development. The objectives of this thesis were thus to identify the possible primary causal agents of tip rot by employing a variety of techniques, including morphological (based on symptom appearance, isolation, and microscopy) as well as molecular approaches, such as sequencing and metabarcoding, followed by a fulfilment of Koch's postulates through pathogenicity testing. In addition, the effect of temperature on the latent period (time lag between infection and tip-rot symptom development) of tip rot was determined.

2. Materials and Methods:

2.1. Tip rot causal agent identification:

Based on the previous pilot study (Nærstad, 2015), we hypothesize that fungi are involved in tip rot of carrots. The identification included several steps:

2.1.1. Carrot source for pathogen identification:

Many samples were studied in this project during 2019/2020, the samples were from various areas in Norway. We got carrots from four fixed fields (i.e. fixed field is one which has historic problem of tip rot were selected by advisors of NLR, and carrot samples were taken while carrots were in the field, at harvest and after storage. The fixed field was also used to take soil samples for plant parasitic nematode identification because some fungi like *Cylindrocarpon* spp. are known to associate with nematodes) and six commercial cold storages (i.e., it means the storages were selected arbitrarily) from Rogaland, Trøndelag, Innlandet and Viken. Selection of fields and cold storages and sampling were caried out in close collaboration with advisors in Norsk Landbruksrådgiving (NLR).

2.1.2. Sample's information:

Carrot and soil sampling from the fixed fields were in five locations in each field as position M1, M2, M3, M4, M5 (Fig. 9A). The fields had a minimum of 1 hectare or more, and it was no relation to the directions in choosing the samples. The samples were taken at three different stages: 1. During mid growing season (June/July 2019-2020) when the carrots reached 50% of the final maturity size or the growth stage of the carrot (BBCH 43-46), those were checked for the presence of tip rot symptom, but at this stage tip rot was not visible, so the carrots were used for DNA extraction to identify the latent infection. Some of the carrots were stored at 4℃ to check if the tip rot will develop from those carrots. 2. At harvesting time (September/ October 2019-2020), the samples were used for both DNA extraction and isolation on culture media. 3. After storage (March/ April 2019-2020): The carrots harvested from fixed fields (September/October) were stored at commercial cold storages until (March/April), those were used for both DNA extraction and isolation on culture media.

In addition to the fixed fields, we got samples from six commercial cold storages in March/April 2019-2020. From each storage a sample of 50 healthy carrots and 50 carrots with tip rot symptoms were sent to NIBIO at Ås. From each, we select 20 healthy and 20 carrots with different tip rot symptoms based on lesions colour (light brown, brown, dark brown, and black) and lesion size (0-2mm, 2-5mm, 5-10mm, 0ver 10mm) and those carrot samples were used for both DNA extraction and isolation on culture media (Fig. 9 B-D).

Fig. 9. Sampling procedures of carrots in four fixed fields; positions (M1 to M5) where carrots were sampled in the field (**A**); selecting carrots for further testing (**B**); washing carrots before isolation (**C**); selecting 20 healthy and 20 symptomatic (tip rot) carrots from each field or storage. For DNA extraction, the 20 carrots were divided into five pseudo replicates (parallels) with four carrots per replication (**D**). Photo (B. Asalf).

2.1.3. Identification by molecular methods:

Mid-season sampling from fixed fields: carrot samples were collected from four fixed fields as mentioned above in the growing season, in June/July 2019-2020. From each field, the local advisory service (NLR, Norsk Landbruksrådgiving) packed 100 carrots, i.e., 20 from each sampling position (M) and sent them to NIBIO at Ås. Then we selected 40 representative carrots and grouped them in two (20 carrots in each). Since there was no symptom of tip rot, isolation was not conducted. For DNA extraction, one group was washed, and the other group was kept unwashed. The washed samples serve to see the microbes in the carrot as endophytes, whereas the unwashed samples were used to see the microbes on and in the carrot. Then we arbitrarily divided the 20 carrots into five pseudo replicates (parallels) with 4 carrots in each and used them for DNA extraction.

Sampling at harvest time: The extension agents from NLR sent samples of 50 healthy carrots and 50 carrots with tip rot from each fixed field and commercial storage to NIBIO at Ås. Then we washed the carrots and selected 20 healthy and 20 with tip rot symptoms. Carrots with tip rot symptom were used for both morphological identification (isolation and incubation) and molecular analysis, whereas healthy carrots were used only for molecular analysis.

Samples carrots from four fixed fields after storage and from six commercial storages: NLR sent 100 carrots, 50 appearing healthy and 50 with tip rot from carrots harvested from the four fixed fields and stored in the commercial storages and arbitrarily selected six commercial storages to NIBIO at Ås. Then they were sorted in the laboratory, and 20 representative carrots were selected from each group. For DNA analysis, we grouped the 20 carrots into five, each with four carrots (The five groups served as replicates for statistical analysis). We did the same grouping for the carrots with tip rot for DNA analysis, and in addition we did isolation and moist incubation from each carrot (20 carrots with tip rot symptoms).

2.1.4. DNA extraction:

Slices were cut from 4 carrots, around 0.5 cm of the taproot and 0.5 cm of the base (tip), from each location. Samples were gathered in Eppendorf tubes and the tubes were labelled by region and year and with tip rot or without and stored at -20°C. For DNA extraction many kits were tried and based on the preliminary results the Fast DNA™ SPIN kit for soil (MP Biomedicals, Santa Ana, California, USA) was chosen. This kit is designed for use with the Fast Prep instruments from MP Biomedicals, plants and animal tissues, bacteria, algae, fungal spores, and other members of a soil population.

In the beginning, samples were placed into 2.0 mL tubes with Lysing matrix E (a mixture of ceramic and silica particles) which efficiently lyse all soil organisms including eubacterial spores and endospores, gram positive bacteria, yeast, algae, nematodes, and fungi. Then MT buffer and Sodium Phosphate Buffer was added, and the tube homogenised (these reagents help to extract the genomic DNA with minimal RNA contamination). After lysis, the samples were centrifuged to pellet soil, cell debris and lysing matrix, then the DNA was purified from the supernatant.

The protocol of Fast DNA soil Kit: https://eu.mpbio.com/media/productattachment/LSO82019-EN-fastDNA-SPIN-kit-for-soil-116560200-Manual.pdf.

2.1.5. Metabarcoding:

The extracted DNA was amplified by PCR by using a metabarcoding approach (16s rRNA for bacteria and ITS1 and ITS2 for fungi/oomycetes) in high throughput sequencing technology. Each DNA fragment, which has a specific sequencing for different species got different barcodes. Those barcodes were compared to previously generated DNA sequences in a reference database from well-characterized species for identification/taxonomic assignment, while the number of reads of each sequence was used to estimate the relative abundance of each species. DNA extraction, PCR, and sequencing were performed on Illumina MiSeq by personnel at NIBIO. That work was not part of this thesis.

2.1.6. Morphological identification:

Many procedures were used to identify the different fungal pathogens that were obtained from carrot samples. The following steps were implemented to identify the morphology of the pathogens:

2.1.6.1. Samples:

All carrots were surface sterilized as follows: 1. carrots were washed thoroughly in tap water several times. 2. The root tips were surface sterilized in 70% ethanol for 1 min, followed by 2-5 min in 0.5% NaOCl solution. 3. All samples were then washed three times with sterile distilled water. 4. After that the tip of the taproot base was carefully split into two parts, from the first part, small pieces of tissue from the inside of the infected taproot base (the margin of the diseased and healthy part of the carrot) were transferred to two agar plates, either Potato Dextrose Agar (PDA) or Potato Carrot Agar (PGA). Then the rest of first part was incubated on wetted sterile filter paper in sealed plastic trays. Distilled

water was added to the filter paper, and the trays were covered with transparent plastic bags to maintain water saturated air in the trays. The trays were incubated at room temperature, and the other half of the taproot base was used for DNA extraction (Fig. 10).

Fig.10. Procedures of isolating carrot samples with tip rot symptoms in agar media and incubating the rest of taproot in moist and at room temperature; splitting the carrot and cutting a piece of the taproot (**A**); isolating piece of taproot in artificial media PGA and PDA (**B**); incubate the rest of taproot on wetted sterile filter paper and at room temperature (**C**). Photo (R. Amin).

2.1.6.2. Culture characterization:

Potato carrot agar (PGA) plates were made by heating 4 L of distilled water until it boiled, then adding 80 g of potato and 80 g of carrot and keeping it boiling for 20 minutes. After that, the water was filtered and divided into eight bottles, 0.5 L each, and then adding 7.5 g (Bacto™ agar) to each bottle, followed by autoclavation. When making potato dextrose agar (PDA) plates, the same procedure was followed, but here we only mixed 39 g potato dextrose agar powder in 1 L distilled water. After autoclaving, the bottles were kept at room temperature until they became lukewarm, and then they were filled in 9 cm plastic Petri plates in a laminar flow cabinet. The plates were kept open there until cooled, then the lid was put on, and the date and name were marked, whereafter the plates were kept sealed in plastic bags at room temperature or in cold room for later use.

2.1.6.3. Microscopy identification:

All samples, either isolated on agar medium or incubated in moist environment, were identified, and described. We checked them under microscope and made slides of the samples. The description of the pathogens was based on type of pathogen (fungus, bacteria), the colony morphology, fungal mycelium, sexual and asexual stage, chlamydospore and sclerotia formation, spore morphology and the type of fungal spores (micro and macro-spore).

2.1.6.4. Using PCR in identification:

Pure isolates of the most frequently found fungi were produced by transferring mycelium and/or spores of each fungus to new plates. Then the pure plate (about 4 weeks old) of each fungus (the selected fungi) was sent for sequencing to identify the genus and species of the fungus.

2.2. Pathogenicity test and latent period:

The preparation for pathogenicity test took around 4 months, and during that time we prepared plant material needed, and figured out the infection method.

2.2.1. Plant material (production of healthy carrots):

On 12 June 2020 four cultivars of carrots (Dailyance UB, Brillyance F1, Namdal F1 and Romance F1 PRES) were sown in pots size (25 L), 25 pots for each cultivar (total 100 pots). The seeds were bought from NORGRO AS (Lier, Norway), and the seeds were not treated with fungicides prior to sowing. In each pot approximately 40 seeds were sown (Fig. 11A).

2.2.2. Agronomic practice:

A soil mix product was used, and it consisted of (80% peat moss H1-H4, 10% peat moss H4-H6 and 10% fine sand), and the pots were covered with a fleece (Fig. 11B). In the first three weeks, the carrots were watered with non-fertilized water and after that they were watered by demand with balanced fertilized water; 50:50 Calcinit (calcium nitrate) and crystalline (Kristalon Indigo) full fertilize both from Yara Norge AS (Oslo, Norway) and had an electrical conductivity of about 1.8-1.9. Weekly four carrots of each cultivar from different pots were picked up and checked for any fungal disease or pest infection and there growth parameters of the carrots (number of leaves, root size and weight) were taken regularly. On 14 October carrots were harvested and packed in plastic bags (cultivar by cultivar) and stored at 0°C with 100% (RH), only healthy appearing carrots of approximately uniform size were stored.

Fig.11. Producing healthy carrots; sowing carrot seeds (four cultivars) each cultivar sowed in 25 pots total 100 pots (**A**); covering the pots with fleece after watering to protect against insect or fungal infection (**B**). Photo (R. Amin).

2.2.3. Selection of the candidate causal agents of tip rot and production of fresh inoculum:

The morphological description of the isolates showed that *Mycocentrospora acerina, Cylindrocarpon* spp., *Fusarium* spp. and *Dictyostelium* spp. were the most frequently isolated and observed fungi from carrots with tip rot symptom and the metabarcoding results also showed that *M. acerina* was the most abundant on carrot samples with tip rot symptom. Based on that we produced three inoculum plates of

each candidate pathogens on PDA culture. The age of the inoculums were around four weeks when we did pathogenicity test.

2.2.4. Development of the inoculation method:

We tried different methods with the selected fungal species to determine which method was the most suitable.

2.2.4.1. Inoculate carrot slices by using agar plug:

The carrots were surface sterilized as described previously, and we cut each carrot in slices, the slices were 1-2 cm in thickness and were placed on wet sterile filter paper in plastic Petri dishes (Fig. 12A). An agar plug (5 mm) with mycelium was placed in the middle of the carrot slice. A lid was put on each Petri dish and thereafter they were placed in sealed plastic boxes and incubated in water saturated air at room temperature. Some other carrots were cut from near the taproot base (about 3 cm, including 1 cm of the taproot), and an agar plug was placed on top (Fig. 12B).

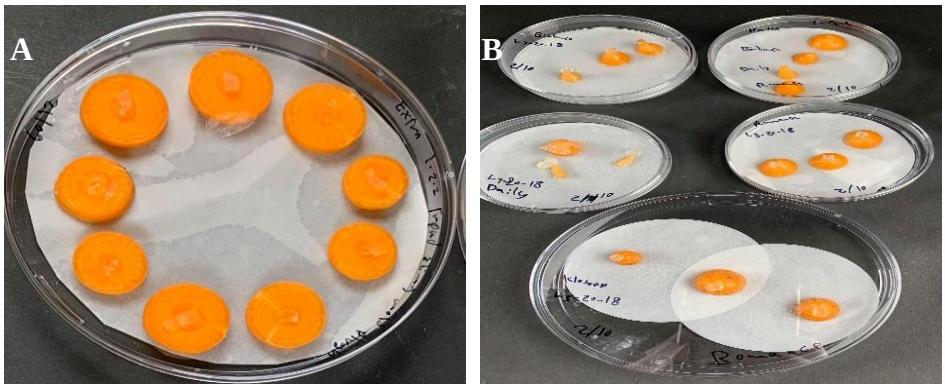

Fig.12. Carrot slices each inoculated with one 5 mm agar piece containing mycelium of *Dictyostelium* spp. (**A**); placing an agar plug (5mm) of *Mycocentrospora acerina* on the taproot (**B**). Photo (R. Amin).

2.2.4.2. Cutting the carrot tips and inoculate the tip by agar plug:

The taproot base was cut off from surface sterilized carrots, i.e., about 0.5 cm of the tip of the taproot and 4 cm of the taproot base. An agar plug (about 5 mm) with mycelium was placed on the taproot of each carrot and incubated in boxes with filter paper and 100 % (RH), and the boxes were stored at room temperature (Fig. 13A-C).

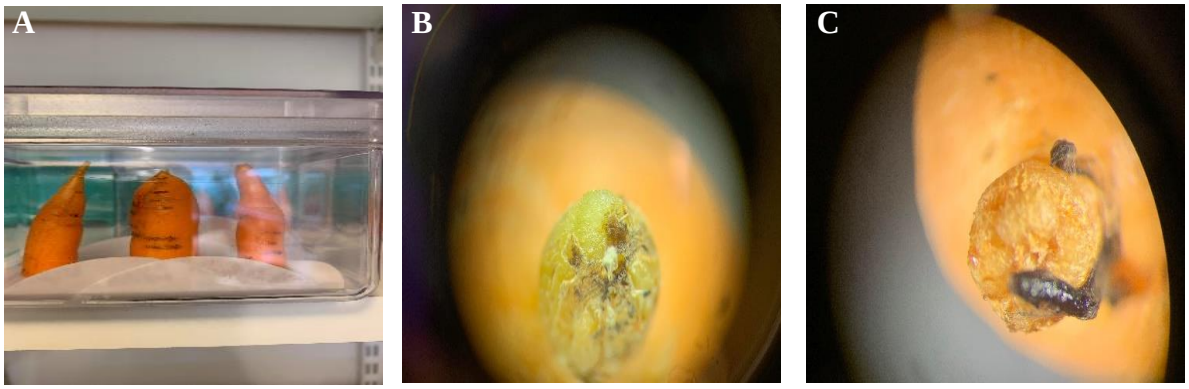

Fig.13. The taproot end of carrots with agar plugs (5 mm) of *Mycocentrospora acerina* (**A**); after inoculation, callus formed on the taproot of some carrots (**B**); the agar plug dried, and the fungus died on some carrots (**C**). Photo (R. Amin).

2.2.4.3. Producing spore of the candidate causal agents on carrot leaves and inoculating carrot slices**:**

We attempted to produce spores of the candidate causal agents of tip rot by inoculating carrot leaves with 5 mm agar plugs of the respective fungi and incubating them on wet filter papers in glass Petri dishes for 8-10 days (Fig. 14 A and B). After that, the carrot slices were inoculated with spores and incubated at room temperature (Fig. 14C).

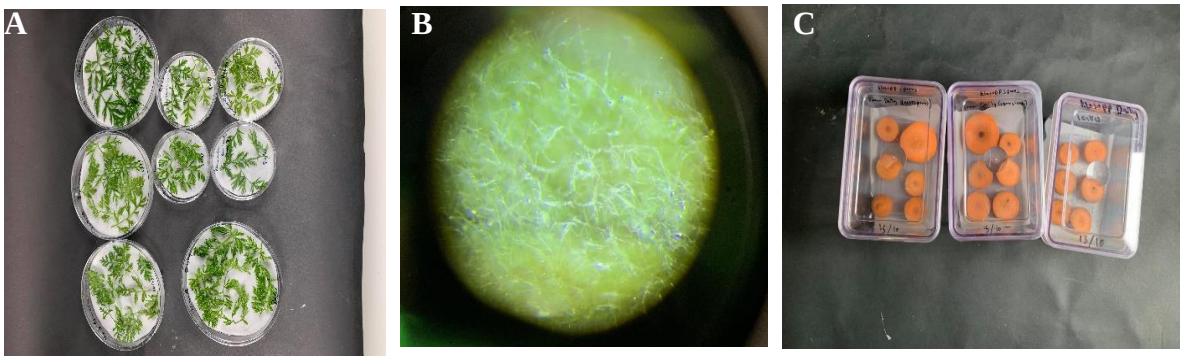

Fig.14. Inoculating carrot leaves with *Mycocentrospora acerina* agar plugs (5 mm) to produce spores (**A**); *Mycocentrospora acerina* sporulating on carrot leaves (**B**); infecting carrot slices with *Mycocentrospora acerina* spores (**C**). Photo (R. Amin).

2.2.4.4. Inoculate the taproot by spore suspension and agar plug:

Five carrots of each cultivar (20 carrots in total) were used, and of these three carrots of each cultivar were inoculated with agar plugs (5 mm) and two were inoculated by spore suspensions (2×10^6 ml^{-1}). The whole carrots without cutting were used. Three to four cm of the taproot was kept, and three different types of injuries in the taproot were attempted, to find the best way to attach the agar plug to it: **1.** The taproot was cut straight off by a knife. **2.** The end of taproot was split by a knife (the end of taproot looked like a V (Fig. 15A)). **3.** Made a hole on the tip of taproot.

The incubation boxes used were transparent and provided with a wet filter paper at the bottom to maintain water saturated air 100% relative humidity, (RH). Additionally, a plastic plug and a metal mesh were used to support the carrots standing vertically and not touching each other (Fig. 15B).

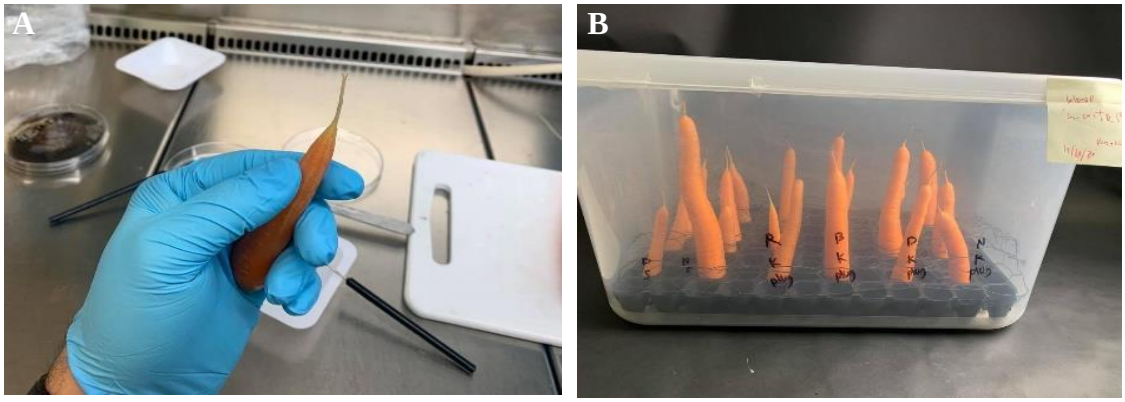

Fig.15. Splitting the taproot of 20 carrots to inoculate 12 of them with an agar plug (5 mm) of *Mycocentrospora acerina*, and the rest with spore suspensions (2×10^6 ml^{-1}) of the fungus (**A**); the inoculated carrots were placed in plastic plugs supported by metal mesh in a box, with 100% relative humidity inside the box, the carrots were incubated at room temperature (**B**). Photo (R. Amin).

2.2.5. Designing the inoculation method for the pathogenicity test:

Based on the results and notes that we got from the methods that we tried above, we decided to use the method from 2.2.4.4 with the whole carrot placed upside down and splitting of the taproot where the agar piece with fungal mycelium was attached. We used four candidate causal agents of tip rot, four cultivars of carrot and three different temperatures. Therefore, we ended up with 30 boxes (as in Fig. 16A), i.e., in each box there were 20 carrots (five carrots of each of four cultivars), there were seven treatments (four pathogens, and one control inoculated with agar and one without, and one control without washing), and there were three storage temperatures (0, 3 and 6°C) and two replicates of each pathogen treatment. The controls (inoculated with agar or without) were not replicated, i.e., one box of each control treatment. However, the control without washing had 3 replicates at each temperature, and each replicate placed in a transparent plastic bag (not box). Material needed for inoculation:

2.2.5.1. Incubation boxes and filter paper:

We used transparent plastic boxes (58 × 39 × 30 cm); the boxes were closed firmly with lids. A wetted filter paper was placed at the bottom under the plastic plug tray that supported the carrots, and relative humidity was maintained 100% in the boxes (Fig. 16A).

2.2.5.2. Plastic plug tray with holes:

In each plastic box there was one plastic plug tray (plug size 32 × 32 × 48 mm) to support the carrots to stand upright and avoid cross contamination between the carrots. The plug tray was cut to fit the boxes (12 × 8 plugs per tray that can hold up to 96 carrots). In addition, we put a tinytag temperature and relative humidity data logger inside one box at each storage room to calculate the temperature and (RH) inside the box. The loggers used were Tinytag Plus 2 TGP 4017 (Gemini, Chichester, UK). Figure

16 B and C illustrates the boxes used for inoculation of the carrots at different temperatures, and it shows the logger inside the box.

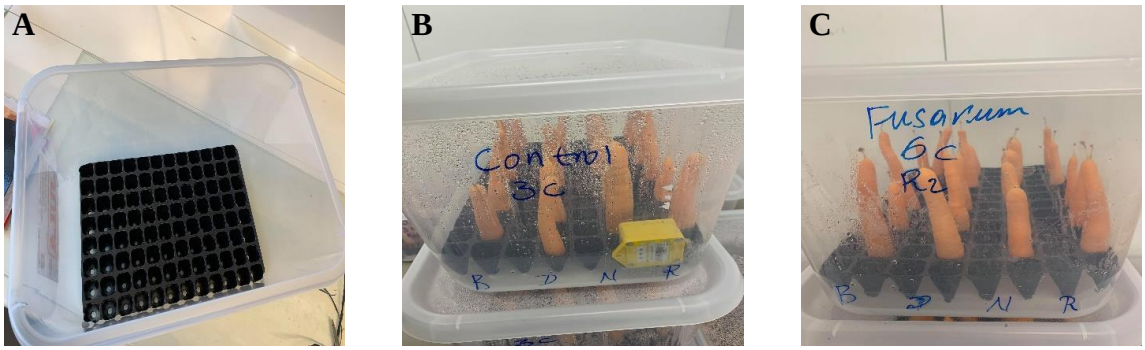

Fig.16. Material used in the inoculation process; white, transparent plastic boxes with filter paper and plastic plug tray (**A**); control box with a logger inside it to calculate temperature in the box (**B**); four carrot cultivars inoculated with *Fusarium* spp. and incubated at 6°C (**C**). Photo (R. Amin).

2.2.5.3. Inoculum production of the candidate fungi:

Pure culture that contains the candidate pathogens were selected from the isolate collections and used for mass production of the inoculum on PDA. All isolates for the candidate pathogens were transferred at same date (15 October 2020) to PDA agar culture, *M. acerina* (L1-20-TR18) and *Fusarium* spp. (L1-20-TR19) had been sampled were from a commercial storage from Viken. While *Cylindrocarpon* spp. (RH19-7) was isolated from a sample in fixed field in Rogaland at harvesting time 2019, and *Dictyostelium* spp. (TG19-3) from a fixed field in Trøndelag.

2.2.5.4. Healthy carrots:

Healthy carrots approximately the same size and without any injury of the four cultivars were used. The total number of carrots was 780 (195 of each cultivar), i.e., 600 carrots were treated with the four candidate pathogens and (positive, negative control), and 180 carrots were 3 replicates of unwashed control at each temperature.

2.2.6. Inoculation:

The inoculation took place on 12 November 2020 by using agar plugs. The inoculated carrots were stored in three cold rooms with three different temperatures at Vollebekk, Ås. The cold rooms were rented from SKP, and temperature were automatically controlled. The room temperatures were recorded during the whole experimental period and were obtained from SKP at the end of the experiments.

2.2.6.1. Inoculation procedures:

Carrots of uniform size and free from damage and contamination were selected, washed, surface disinfested and the longest taproot was cut about 2 cm above the main carrot root, then split in V-shape of 3 mm length as showed in Figure 15A above, then the inoculum on agar plate was cut to 5mm (the

agar contain fungal mycelium and spore for each of the pathogens) and placed between the split roots. The detailed steps of inoculation were as follows:

- All boxes (30 boxes) were prepared (filter paper+ plastic plugs+ distilled water), and 150 carrots from each cultivar were selected (same size).
- Additionally, 180 carrots (45 carrots of each cultivar) picked up and putted in bags as unwashed control, 3 replicates at each room temperature; 3 temperatures × 3 reps × 5 carrots × 4 cultivars.
- All carrots were washed carefully in tap running water several times, and surface sterilized by the following sterilization protocol, which was: Dipping in 70% ethanol for 30-60 sec followed by placing the samples in 0.5% sodium hypochlorite for 2-5min. After that all carrots were rinsed in doubled autoclaved water.
- We picked up 15 healthy carrots from each cultivar and placed them in 3 boxes as control, one box for each temperature (negative control).
- From the remaining 135 carrots of each cultivar, we selected carrots randomly and cut the tip of the taproot approximately 2 cm from the mother root. The taproot was then split (about 3 mm depth) to make place for an agar plug and support it so it did not fall off. Then all carrots were covered by clean paper to maintain it clean and to avoid the dryness (during inoculating carrots).
- We selected 15 (wounded) carrots from each cultivar and used clean agar plugs without mycelium on each carrot, those carrots were placed in 3 boxes. One box for each temperature (those boxes were considered as positive controls).
- The remaining carrots (5 carrots from each cultivar) were placed in the remaining 24 boxes, and carrots in each of 6 boxes were inoculated with a candidate pathogen, and the boxes were distributed at three different temperatures.

2.2.6.2. Overview of incubation in the storage:

The inoculated carrots incubated firstly at room temperature (19 ± 0.2℃) with light for 24 hours, and after that they have been moved to three different rooms 0, 3, and 6℃ with light. The carrots were stored at each temperature as it described below and see (Fig. 17):

- 2 boxes inoculated with *Mycocentrospora acerina* and each box include 20 carrots (4 cultivars × 5 parallels).
- 2 boxes inoculated with *Cylindrocarpon* spp., and each box include 20 carrots (4 cultivars × 5 parallels).

- 2 boxes inoculated with *Fusarium* spp. and each box include 20 carrots (4 cultivars × 5 parallels).
- 2 boxes inoculated with *Dictyostelium* spp., and each box include 20 carrots (4 cultivars × 5 parallels).
- 1 box considered as a control (washed carrots only without splitting the taproot), it contains 20 carrots (4 cultivars × 5 parallels).
- 1 box considered as control (washed carrots with splitting the taproot and putting pure agar plug on it), it contains 20 carrots (4 cultivars × 5 parallels).
- 3 replicates of bags considered as unwashed control: each bag contains 20 carrots (4 cultivars × 5 parallels).

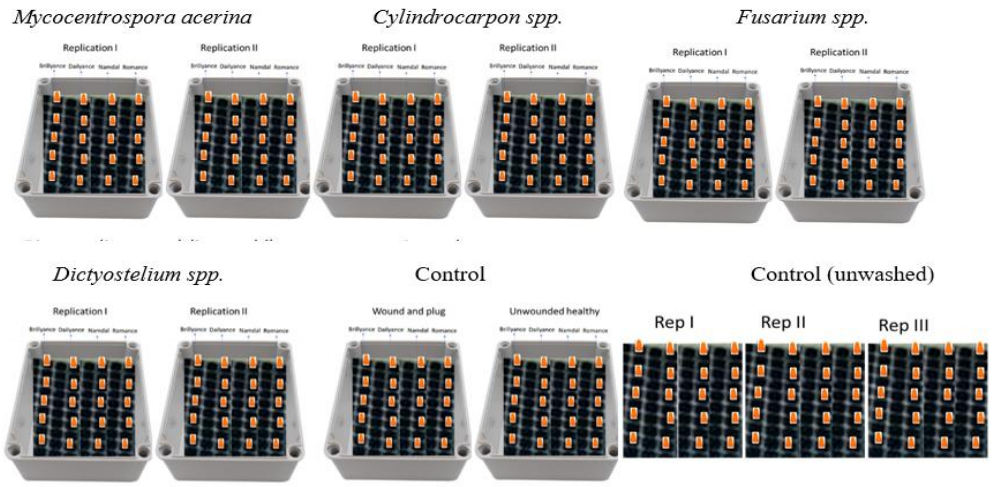

Fig .17. Overview of the carrots stored at each room temperature. (Photo B. Asalf).

2.2.7. Disease assessments:

Assessments were made for the length of the latent period (number of days from inoculation to first appearance of symptoms), disease incidence (the number of carrots showed tip rot symptom that is lesion developed at least 2mm from the point of inoculation) and disease severity (how deep the lesion extend from the point of inoculation in millimetre (mm). Assessments for latent period was made every 2 days until some symptoms appeared on more than 50% of carrots. Disease severity was recorded at every 7 days interval (unless extended due to COVID-19 lock down and restrictions). At the same time, we registered how the symptoms looked like to compare them with the symptoms on the original carrots that the inoculum was isolated from and to show the difference between four candidates. In addition, we recorded other observable variations among pathogens and cultivars for example callus formation,

regrowth of roots and leaves, and presence of contamination of other pathogens during the experiment. The disease registration took place from 16 November 2020 until 17 March 2021.

2.2.8. Re-isolation of the pathogen:

We selected representative samples with different symptoms in appearance (dry and wet) or in colour. The re-isolation process included checking carrot tissue, and then re-isolating the pathogen on PDA, PGA media. Then after incubating the rest of the carrot in moist and at room temperature. One week later the isolates and incubated samples were checked, and the plates were purified. Then the fungal growth and culture characters were compared with the original inoculum that was used in pathogenicity test.

2.2.9. Data analysis and statistics:

The latent period, disease severity and AUDPC data was subjected to analysis of variance (ANOVA) with the mixed effect ANOVA to see the effect of storage temperature, cultivar, and storage temperature × cultivar on tip rot development. The mean separation or grouping information was done by using Tukey pairwise comparisons method and 95% confidence (p-value = 0.05) in MINITAB 19. In addition, Area under disease progress curve (AUDPC) was calculated based on disease severity. Which was the length of lesion (tip rot) from the point of inoculation to the taproot estimated in (mm). On the control carrots that were not inoculated with pathogens, there was not typical tip rot symptom, so the data from the uninoculated control carrots were not included in the data analysis, because the disease severity and incidence data values were zero.

3-Results:

3.1. Tip rot causal agent identification:

3.1.1. Description of tip rot symptoms (Koch's postulate step 1):

To correctly identify the causal agent of tip rot, the first step was to describe the symptoms and signs of the disease. Tip rot symptom ranges from light brown, dark brown and black and it was either wet or and dry texture (Fig. 18). The symptoms also slightly vary between unprocessed carrots (that was not washed and polished) and processed carrots that pass through the washing, polishing, and packaging process in the package house. Most of the unprocessed carrot with the long thin taproot attached showed brown to blackish symptom (Fig. 18A), whereas most of processed carrot (washed, the long thin taproot removed and polished) showed a characteristics light and glassy type symptom, see carrot numbered from 1 to 11 in figure 18B.

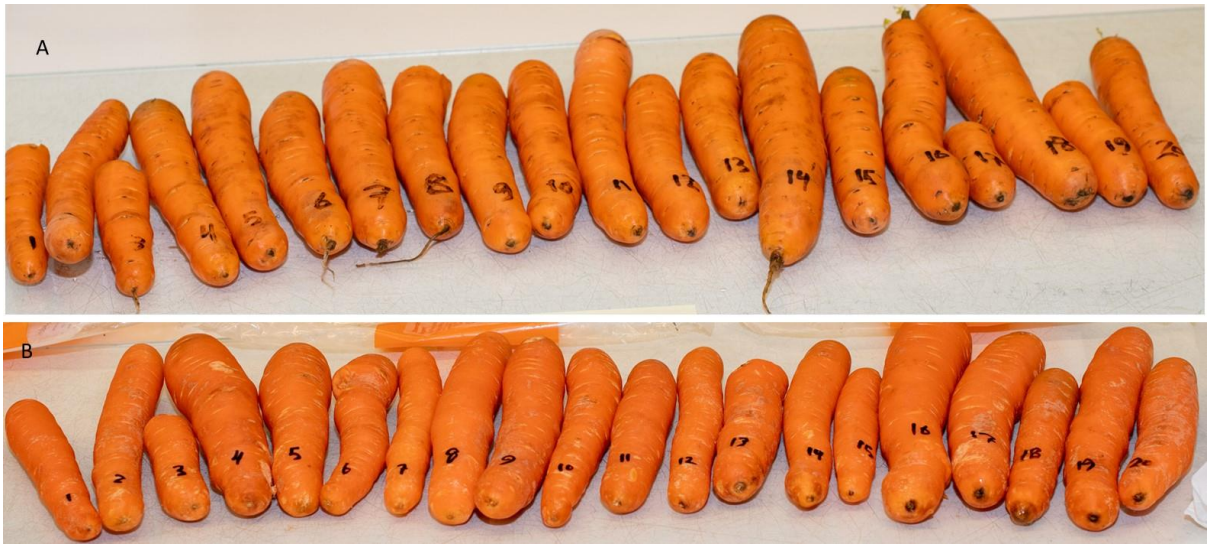

Fig.18. Different tip rot symptoms after sorting, (samples were collected from the same field); Carrots with tip rot symptom sampled before they were washed, polished, and packed (**A**); Carrots samples with light, brown and black tip rot symptom after washed, polished, and packed for marketing (**B**). (Photo B. Asalf).

3.2. Isolation in pure culture and molecular identification (Koch's postulate step 2):

3.2.1. Metabarcoding results:

The metabarcoding results showed that *Mycocentrospora acerina* was the most abundant pathogen in samples with tip rot symptoms. In addition to *M. acerina*, there were other fungi pathogenic to carrots that showed up in the metabarcoding analysis. For instance, *Fusarium* spp., *Rhizoctonia* spp., *Sclerotinia* spp., *Botrytis* spp. and *Ilyonecteria*/ *Cylindrocarpon* spp.

3.2.2 Morphological identification:

Based on microscopic identification of fungal morphology from fungi grow on artificial media (PDA and PGA) and in carrot incubated in moist, 15 different fungi were identified from carrots with tip rot symptom. In addition, we could not identify many fungi so those were registered as unknown. The abundant fungi associated with tip rot identified from carrots sampled at harvesting time were presented in figure 19 and those identified after storage in cold rooms were presented in figure 20. The distribution of pathogens varied among storages (Figs. 19 and 20), some storages had only one pathogen whereas others had more than one pathogen, for example from T.H20.TR more than four fungi were identified (Fig. 19) and from L3.20 more than six fungi were identified (Fig. 20). At harvesting samples were isolated in September/ October 2019-2020, and after storage samples were isolated in March/April 2019-2020.

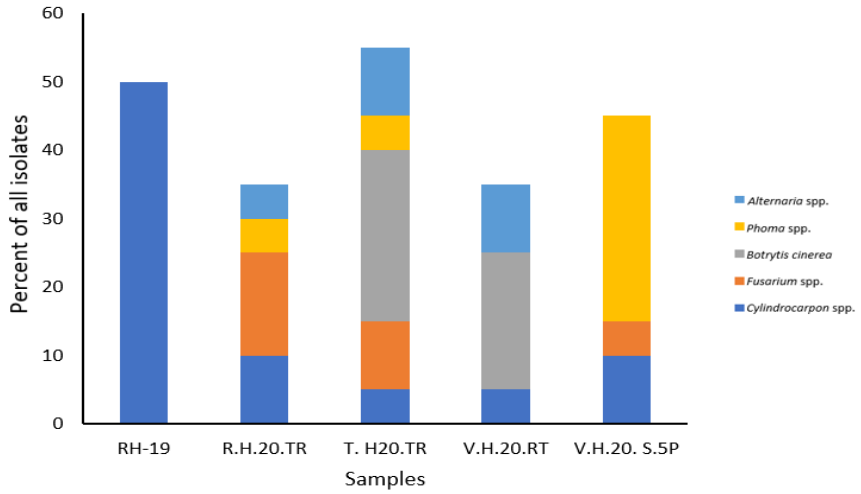

Fig.19. The identified potential pathogens after isolating and incubating samples (at time of harvest) from five fields: Rogaland 2019 (RH-19), Rogaland 2020 (R.H.20.TR), Trøndelag 2020 (T. H20.TR), Viken 2020 (H.20.RT), Viken 2020 (V.H.20. S.5P). Except from RH-19 (8 carrots used for isolation), the other locations isolation was done from all the 20 carrots with tip rot symptom that were used for DNA analysis.

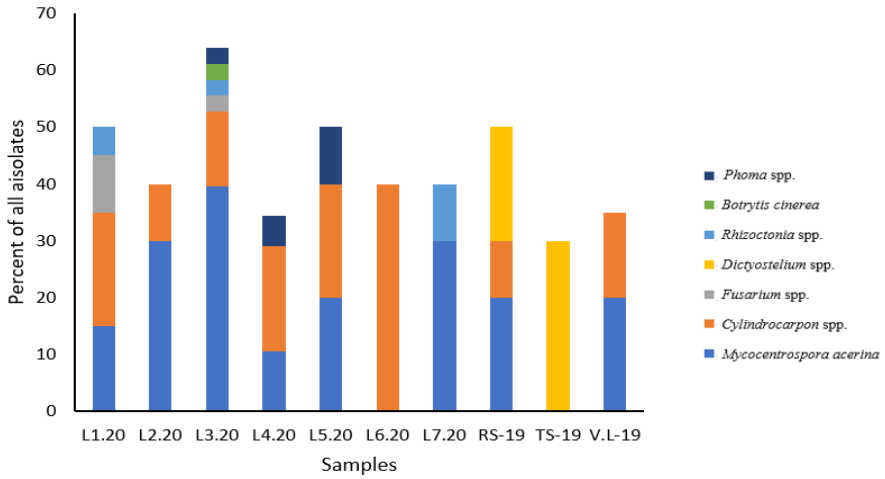

Fig.20. The identified potential pathogens after isolating and incubating samples from six commercial storages and carrots harvested from the four fixed fields and stored in four commercial storages respectively: Viken 2020

(L1.20), Rogaland 2020 (L3.20), Rogaland 2020 (L4.20), Innlandet 2020 (L5.20), Innlandet 2020 (L6.20), Viken 2020 (L7.20), Viken 2020 (L2.20), Rogaland 2019 (RS-19), Trøndelag 2019 (TS-19), Viken 2019 (V.L-19). Except from L3.20, L4.20 (38 carrots used for isolation) and L1.20, V.L-19 (20 carrots used for isolation), the other locations totally included 10 isolates of different fungi.

3.2.3. Candidate pathogens and inoculum culture characters:

Based on metabarcoding and morphological results, four of the most frequent pathogens (*Mycocentrospora acerina*, *Cylindrocarpon* spp., *Dictyostelium* spp., and *Fusarium* spp.) were selected. Then a pure inoculum was produced on (PDA) culture media and their symptoms on carrot tissue and characters on agar media were noted to fulfil the Koch's postulate:

1. *Mycocentrospora acerina:* The symptoms on carrot tissue were wet tissue with dark black (Fig. 21A) and after isolating it on PDA, the agar plate characters were circular growing form, filamentous margin, elevation was convex, and the plate was black from both sides (Fig. 21B). See figure 40 C and D in the appendix. In addition, the chlamydospores and spores of *M. acerina* were observed easily on PGA media.

2. *Cylindrocarpon* spp.: The symptoms on carrot tissue were brown to dark brown tip (Fig. 21C), and after isolating it on PDA the characters were circular growing form, filamentous margin, elevation was convex, and the plate was brown from the top and dark brown from bottom sides (Fig. 21D).

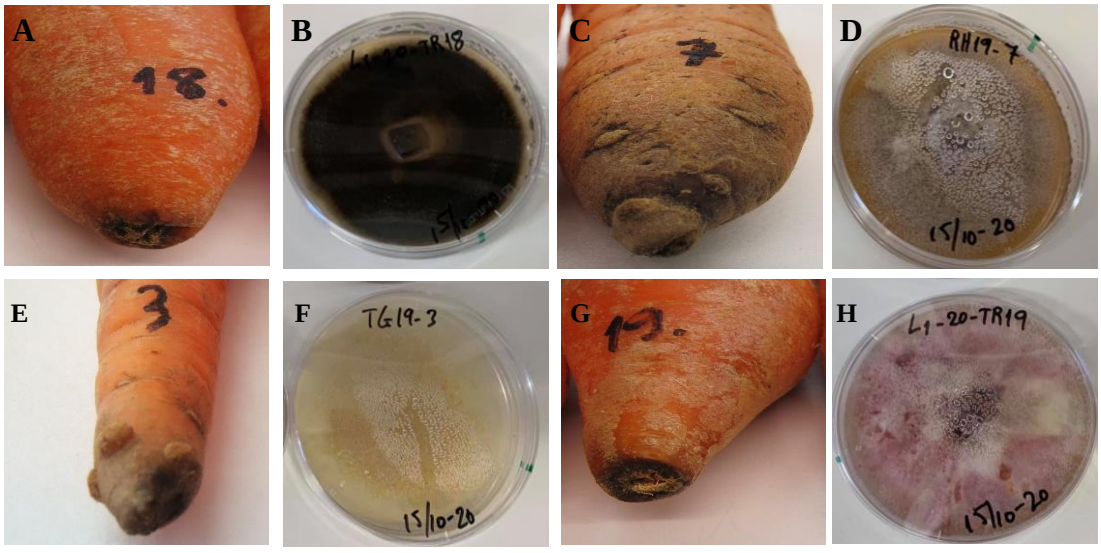

Fig.21. The original carrots that candidate fungi were isolated from with agar plates of each fungi; the carrot that *M. acerina* isolated from **(A**); *Mycocentrospora acerina* on PDA (**B);** the carrot that *Cylindrocarpon.* spp. isolated from (**C**); *Cylindrocarpon* spp. on PDA (**D**); the carrot that *Dictyostelium.* spp. isolated from (**E**); *Dictyostelium* spp. on PDA (**F**); the carrot that *Fusarium.* spp. isolated from (**G**); *Fusarium* spp. on PDA (**H**). (Photos A, C by V. Hong Le and the rest by R. Amin).

3. *Dictyostelium* spp.: The symptoms on carrot tissue were light brown to brown and the tip was wet (Fig. 21E), then after isolating it on PDA the characters were irregular growing form,

elevation was raised, and the plate was light brown from both sides. (Fig. 21F). See figure 40 C and D in the appendix.

4. *Fusarium* spp.: The symptoms on carrot tissue were dark brown and the tip was dry (Fig. 21G), then after isolating it on PDA the characters were circular growing form, elevation was convex, margin was filamentous, and the plate was red/pink from both sides. (Fig. 21H). See figure 40 C and D in the appendix.

3.3. Reinoculation and disease development (Koch's postulate step 3):

After inoculation of the candidate pathogen on healthy carrot, tip rot development was measured by assessing the latent period, disease incidence and area under disease progress curve (AUDPC) based on disease severity.

3.3.1. Latent period:

Room temperature: The mean temperature of the rooms programmed 0, 3 and 6℃ were 0.5℃ (0.5), 3.4℃ (0.5) and 6.1℃ (0.5), respectively. Values in the bracket are standard deviation. While the loggers showed that the temperature in the boxes at rooms programmed 0, 3, 6℃ were 6 + 0.2℃, 3 + 0.4℃, and 0 + 0.3℃, respectively and the relative humidity (RH) was 99.98% in the boxes. There was a slight increase in temperature in each room, but it was within the range that we were informed by SKP (i.e. 0 + 1℃, 3 ± 1℃ and 6 ± 1℃).

-The effect of storage temperature, cultivar, and temperature × cultivar on the latent period varied for each pathogen. *Mycocentrospora acerina*: The latent period was significantly different among storage temperature ($P < 0.001$) and among cultivars ($P = 0.014$), but there was no interaction effect of temperature and cultivar ($P = 0.779$) (Table 1).

Cylindrocarpon spp.: The latent period was significantly different among storage temperature and cultivars ($P = 0.035$ and $P = 0.013$) respectively, and there was a clear interaction between temperature and cultivar ($P < 0.001$) (Table 1).

Dictyostelium spp.: The latent period was significantly different among storage temperature ($P = 0.029$), but there was no difference among cultivars and no interaction effect of temperature and cultivar ($P = 0.056$ and $P = 0.059$) respectively (Table 1).

Fusarium spp.: The latent period was significantly different among storage temperature ($P < 0.001$) and among cultivars ($P = 0.021$), but there was no interaction effect of temperature and cultivar ($P = 0.236$) (Table 1).

For all pathogens, the latent period at 6℃ was shorter than at 3 and 0℃ (Table. 1). In addition, cultivars influenced disease development and cultivar susceptibility was dependent on the treatment (pathogen). Cultivars 'Namdal' and 'Brillyance' had a latent period of about 6 and 7 days at 6℃ for *M. acerina*

while Namdal had the shortest latent period about 6 days for *Cylindrocarpon* spp. and about 7 days for *Fusarium* spp. (Table. 1). Romance had the shortest latent period about 7 days for *Dictyostelium* spp. (Table. 1).

Table.1. Effect of temperature and cultivar on latent period of *Mycocentrospora acerina*, *Cylindrocarpon* spp., *Dictyostelium* spp. and *Fusarium* spp.

Temperature (°C)	Carrot cultivar	Latent period (number of days from inoculation until tip rot symptom observed)			
		Mycocentrospora. acerina	*Cylindrocarpon* spp.	*Dictyostelium* spp.	*Fusarium* spp.
0	Brillyance	10.3 ab	11.5 a	28.3 a	22.9 a
0	Dailyance	12 a	9.9 ab	25.8 ab	17.4 abcd
0	Namdal	10.4 ab	11.2 a	24 ab	11.3 abcd
0	Romance	11 ab	11 a	20.5 ab	21.1 ab
3	Brillyance	7.9 bcd	10.2 ab	24.7 ab	13.4 abcd
3	Dailyance	9.2 abc	10.8 ab	23.4 ab	15.7 abcd
3	Namdal	8.5 abcd	9.8 ab	16.9 ab	12.2 abcd
3	Romance	10 ab	11.8 a	27.2 a	18.6 abc
6	Brillyance	6.9 cd	11.8 a	24.7 a	7.3 cd
6	Dailyance	8.4 abcd	11.5 a	21 ab	9.2 bcd
6	Namdal	6.2 d	7.8 b	18.4 ab	6.6 d
6	Romance	7.6 bcd	8.2 b	7.3 b	8 cd

Means in the same column for each pathogen that do not share a letter are significantly different (P-value ≤ 0.05).

3.3.2. Disease incidence and symptoms development:

There was a variation in disease incidence of each candidate pathogen between storage temperature and cultivars. The incidence of *M. acerina* appeared in less days than *Cylindrocarpon* spp. and *Fusarium* spp. while *Dictyostelium* spp. needed more many days to reach a 100% the incidence. The incidence reached 100% in short period of time after inoculation for carrots incubated at 6°C compared to the same cultivar inoculated with the same pathogen and incubated at 0°C (Figs. 22-25).

Disease incidence of *M. acerina* at 0°C on assessment date (20.11.2020) was (0, 0, 20 and 30 %), while on the same date, the incidence was (30, 60, 90 and 70%) at 3°C, and (100, 40, 80 and 100 %) at 6°C for Romance, Dailyance, Brillyance and Namdal, respectively (Fig. 22).

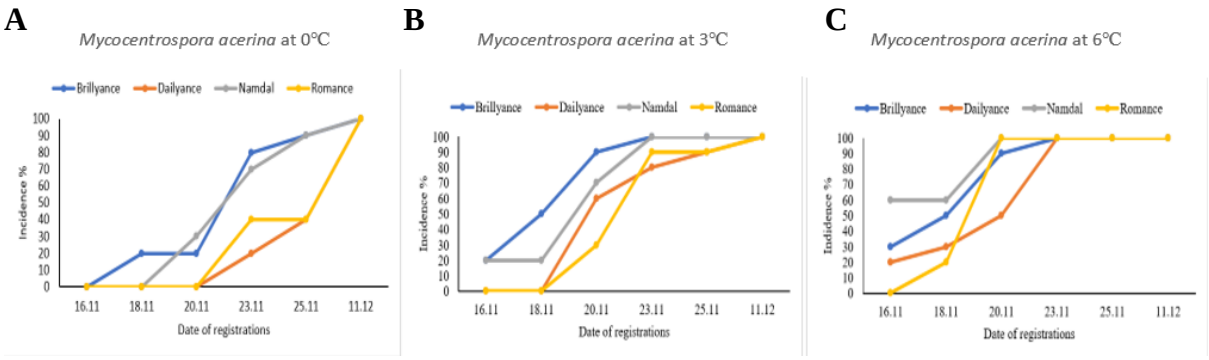

Fig.22. Incidence (%) of *Mycocentrospora acerina* by dates of assessment at 0°C, (**A**); 3°C, (**B**); and 6°C (**C**).

Disease incidence of *Cylindrocarpon* spp. at 0°C on assessment date (25.11.2020) was (60, 60, 40 and 100%), while on the same date, the incidence was (50, 50, 60 and 90%) at 3°C, and (100, 60, 80 and 100%) at 6°C for Romance, Dailyance, Brillyance and Namdal, respectively (Fig. 23).

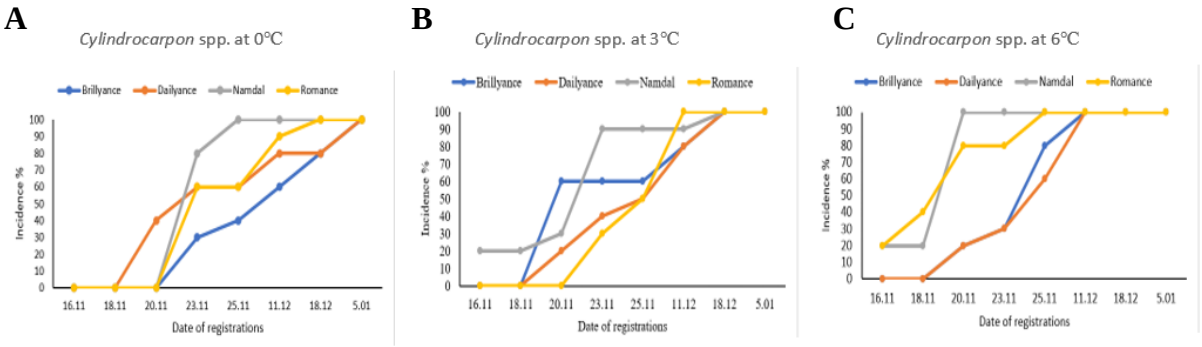

Fig.23. Incidence (%) of *Cylindrocarpon* spp. by dates of assessment at 0°C, (**A**); 3°C, (**B**); and 6°C (**C**).

Disease incidence of *Dictyostelium* spp. at 0°C on assessment date (18.12.2020) the incidence was (100, 30, 20 and 100%), while on the same date, the incidence was (100, 50, 20 and 100%) at 3°C, and (80, 30, 50 and 100%) at 6°C for Romance, Dailyance, Brillyance and Namdal (Fig. 24).

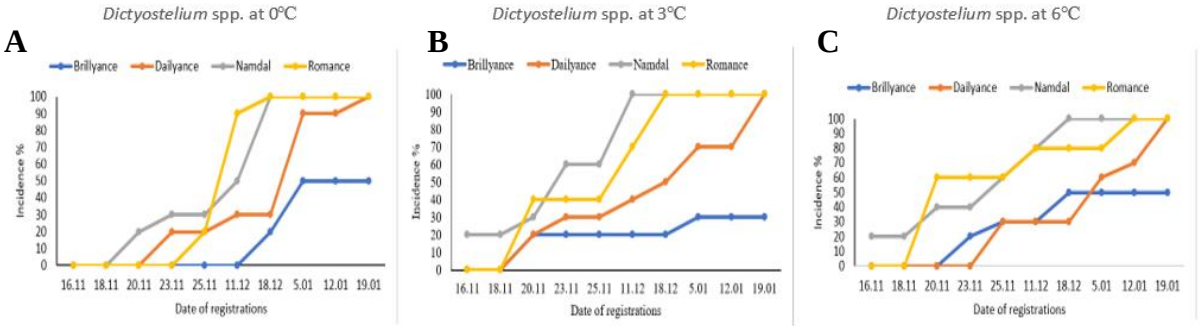

Fig.24. Incidence (%) of *Dictyostelium* spp. by dates of assessment at 0°C, (**A**); 3°C, (**B**); and 6°C, (**C**).

Disease incidence of *Fusarium* spp. at 0°C on assessment date (23.11.2020) was (30, 50, 30 and 70%), while on the same date, the incidence was (30, 60, 60 and 90%) at 3°C, and (90, 60, 90 and 100%) at 6°C for Romance, Dailyance, Brillyance and Namdal, respectively (Fig. 25).

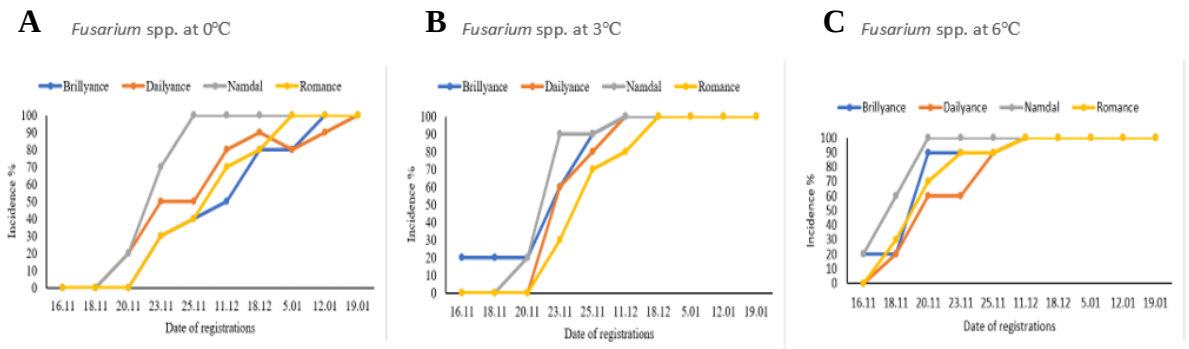

Fig.25. Incidence (%) of *Fusarium* spp. by dates of assessment at 0°C, (**A**); 3°C, (**B**); and 6°C, (**C**).

3.3.3. Description of the symptom on inoculated carrots:

The symptoms were changing as the infection progresses over time. At the start of the infection the symptom looks like similar light to brownish but as the disease progresses the discoloration changed and appear brown to blackish. As the disease progress and infection advanced from the tip of the thin taproot to the mother root, each pathogen developed different symptoms. The most dominant symptoms of the pathogens were as follows: **1.** *Mycocentrospora acerina*: The symptoms were first light brown near the point of inoculation after 4 days of inoculation in all three temperatures. Then after 2 weeks the symptoms were dark brown to black under all temperatures. After 4 weeks of inoculation, black discoloration started to appear at 6°C, whereas in the fifth week following inoculation black symptoms started to appear at 0 and 3°C as well (Fig. 26A). At the end of the experimental period, symptoms were either black and wet, or black and dry (Fig. 26B).

Fig.26. The variation in *Mycocentrospora acerina* development on inoculated carrots and incubated at 3°C (**A**) and 6°C (**B**). (Photo B. Asalf).

2. *Cylindrocarpon* spp.: After 4 days light brown discoloration started to appear on some carrots at all temperatures. Then the symptoms changed to brown under 0℃ and dark brown under 3 and 6℃ in 4 weeks. After 8 weeks, the carrots at 0℃ had dark brown symptoms, and dark brown to black at 3℃ (Fig. 27A), and 6℃ (Fig. 27B). In the end of experimental period, the most dominant symptoms were brown, and dark brown/ black under all temperatures.

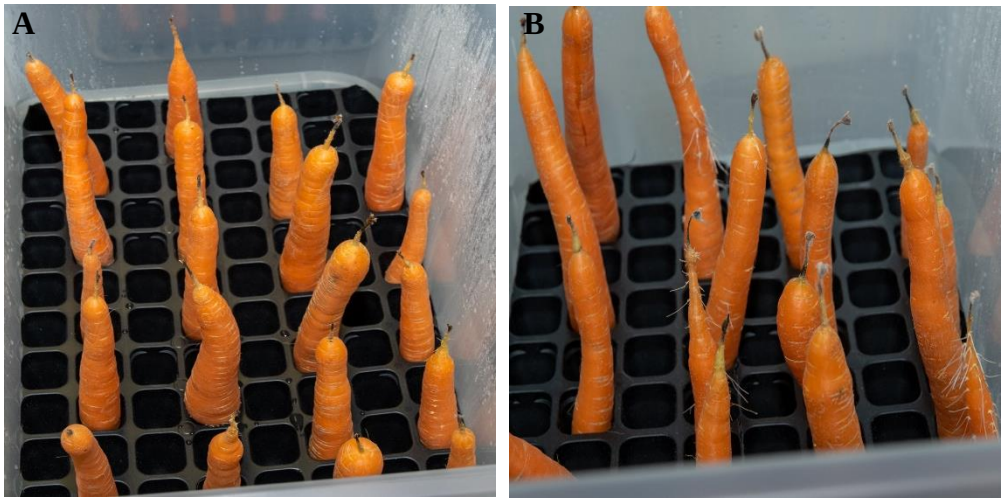

Fig.27. The variation in *Cylindrocarpon* spp. development on inoculated carrots and incubated at 3°C (**A**) and 6°C (**B**). (Photo B. Asalf).

3. *Dictyostelium* spp.: Four days after inoculation, light brown symptom observed on carrots at 3 and 6℃, whereas the symptoms appear at 0℃ after 8 days. After 7 weeks the symptoms became brown to dark brown only at 3 and 6℃ (Fig. 28) A and B. The lesions on carrots under 0℃ got brown to dark brown after 12 weeks. The final symptoms were light dark brown.

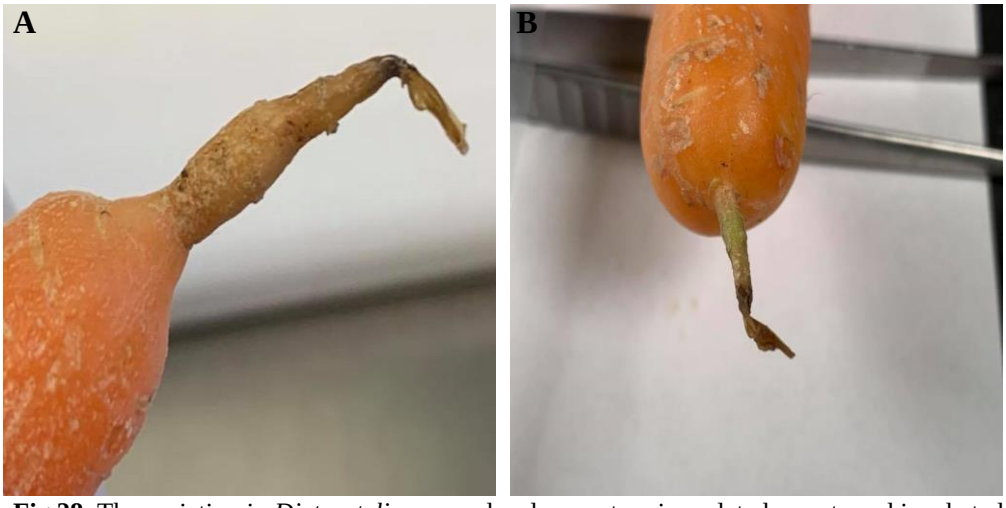

Fig.28. The variation in *Dictyostelium* spp. development on inoculated carrots and incubated at 3°C (**A**) and 6°C (**B**). Photo (R. Amin).

4. *Fusarium* spp.: The initial symptoms appeared as light brown near the inoculation point on the taproot within 4 days of inoculation at all temperatures. After 4 weeks, the rot became dry and brown at all temperatures. At the end, the lesions were either light brown dry or brown to black dry (Fig. 29).

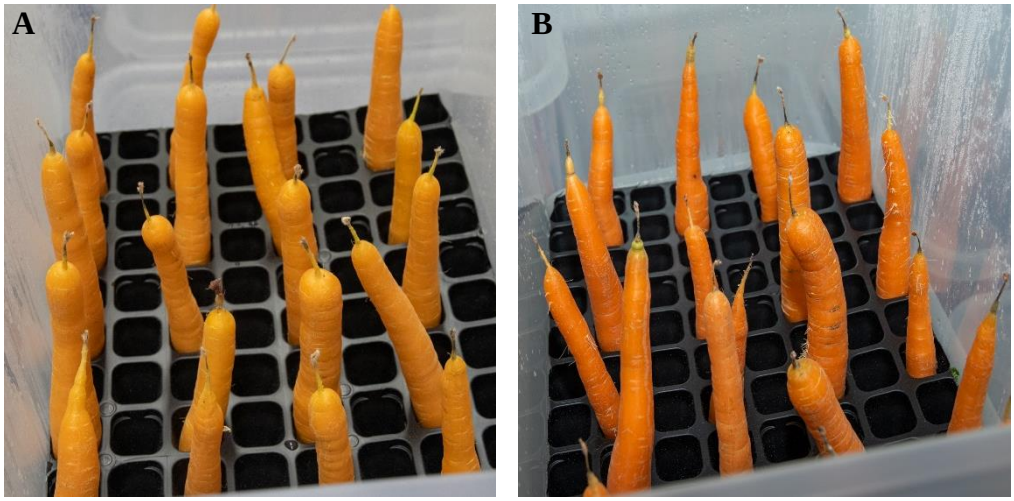

Fig.29. The variation in *Fusarium* spp. development on inoculated carrots and incubated at 3°C (**A**) and 6°C (**B**). (Photo B. Asalf).

3.3.4. Disease severity: The effect of temperature, cultivar, and temperature × cultivar on the AUDPC:

The results showed that the effect of factors storage temperature, cultivar and storage temperature × cultivar on AUDPC based on disease severity varied among the pathogens as follows:

Mycocentrospora acerina: AUDPC was significantly different among storage temperature ($P < 0.001$), and cultivar ($P = 0.05$) but there was no interaction effect of temperature and cultivar ($P = 0.198$). *Mycocentrospora acerina* disease development, which was presented as AUDPC value, was slowest for cultivar Dailyance stored at 0℃, whereas the disease development was the faster for cultivar Brillyance stored at 6℃ (Table. 2).

Cylindrocarpon spp.: AUDPC was significantly different among storage temperature and cultivars ($P < 0.001$ for both), and there was also an interaction effect of temperature and cultivar ($P = 0.001$). *Cylindrocarpon* spp. disease development, which was presented as AUDPC value, was slowest for cultivar Brillyance stored at 0℃, whereas the disease development was the faster for cultivar Namdal stored at 6℃ (Table. 2).

Dictyostelium spp.: AUDPC was significantly different among storage temperature and cultivars ($P = 0.020$ and $P < 0.001$ respectively), and there was also an interaction effect of temperature and cultivar ($P = 0.022$). *Dictyostelium* spp. disease development, which was presented as AUDPC value, was

slowest for cultivar Brillyance stored at 0°C, whereas the disease development was the faster for cultivar Romance stored at 6°C (Table. 2).

Fusarium spp.: AUDPC was significantly different among storage temperature and cultivars ($P < 0.001$ and $P = 0.005$ respectively), but there was no interaction effect of temperature and cultivar ($P = 0.421$). *Fusarium* spp. disease development, which was presented as AUDPC value, was slowest for cultivar Brillyance stored at 0°C, whereas the disease development was the faster for cultivar Namdal stored at 6°C (Table. 2).

Area under disease progress curve based on disease severity (mm lesion size) was calculated for each pathogen at each storage temperature, and it showed the effect of cultivar and storage temperature at each pathogen. The (Table. 2) is for: disease severity expressed as AUDPC, and it shows the severity of tip rot for each pathogen at each room temperature and cultivar.

Table. 2. Disease severity expressed as AUDPC, which reveals the severity of tip rot for each pathogen at each room temperature and cultivar.

Area under disease progress curve[1]					
Storage temperature (°C)	Carrot cultivar	Pathogens[2]			
		Mycocentrospora acerina	*Cylindrocarpon* spp.	*Dictyostelium* spp.	*Fusarium* spp.
0	Brillyance	1717.1 c	269.7 e	59.2 e	499.5 c
0	Dailyance	1627.7 c	544.7 bcde	140.9 de	569.9 bc
0	Namdal	1794.6 bc	777.9 bc	466.4 abc	817.4 bc
0	Romance	1664.5 c	620.3 bcde	227.5 cde	743.9 bc
3	Brillyance	2041.4 abc	477.7 cde	116.8 de	834.1 bc
3	Dailyance	1895 bc	502.4cde	169.5 cde	963.3 abc
3	Namdal	1787.6 bc	742.9 bcd	688.1 a	1028.8 abc
3	Romance	1855.1 bc	375.2 de	374.8 bcd	677.4 bc
6	Brillyance	2425.8 a	542.3 bcde	214.7 cde	929.4 abc
6	Dailyance	1976.1 abc	518.9 bcde	104.2 de	1053.7 ab
6	Namdal	2421.1 a	1246.1 a	473.5 abc	1398.6 a
6	Romance	2245.7 ab	882.9 ab	559.5 ab	992.5 abc

[1]AUDPC estimated based on disease severity (mm) as cumulative weekly assessment.

[2] Means that do not share a letter are significantly different ($P \leq 0.05$).

3.3.5. Callus formation:

Callus formation was observed at 3 and 6°C but not at 0°C. Callus formation started at 6°C after 54 days of inoculation, whereas at 3°C, it was first observed after 98 days of inoculation. There was no significant variation in forming callus between cultivars; however there was significant variation in callus size among the pathogens and specially after inoculation with *Fusarium* spp. large callus formations appeared (Fig. 30A). There was no callus formation for *M. acerina* at 3°C while only two carrots formed callus at 6°C with small callus sizes. The other pathogens frequently formed callus at 6°C (Fig. 30B). For example, for 'Romance' 70, 60 and 60% of the carrots inoculated with *Dictyostelium* spp., *Fusarium* spp. and *Cylindrocarpon* spp., respectively, and stored at 6°C formed callus. Whereas at 3°C the highest callus formation incidence was for 'Romance', where 100 and 80% of the carrots inoculated with *Fusarium* spp. and *Cylindrocarpon* spp. respectively, formed callus (Fig. 30B).

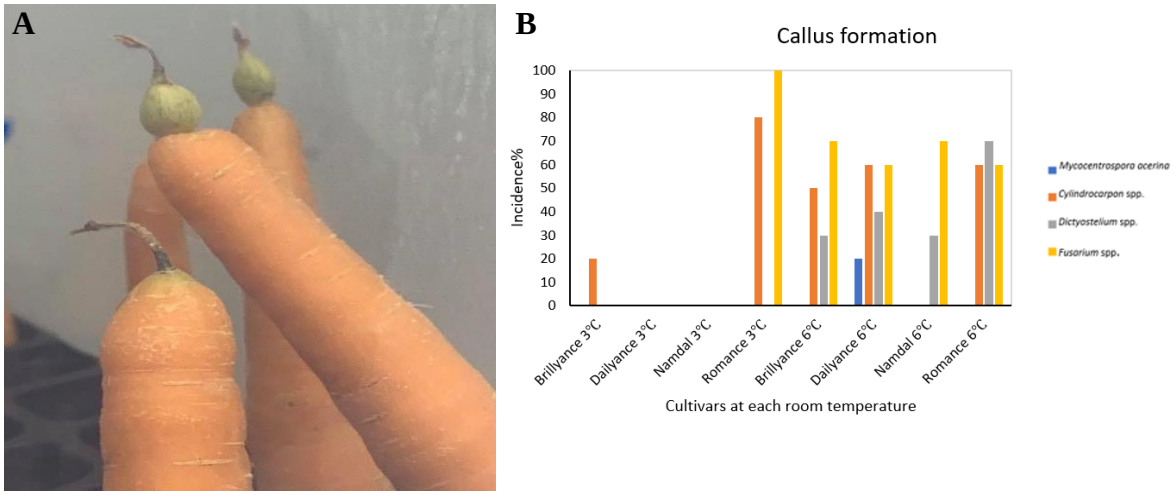

Fig.30. Callus formation at different temperatures for the four pathogens and cultivars; callus formation on carrots infected with *Fusarium* spp. and stored at 6°C (**A**); callus formation at different temperatures /pathogens/cultivars (**B**). Photo (R. Amin).

3.4. Re-isolation of the pathogen and fulfilling (Koch's postulate step 4):

Two carrots with different symptoms, mainly with dry and wet type tip rot were selected arbitrarily from each room for each pathogen and brought to the laboratory for further symptom description before and after splitting the carrot. Then the pathogen was re-isolated, and the rest of the tissue was incubated in water saturated air at room temperature. The results that we got from each treatment at each temperature are organized as follows:

3.4.1. *Mycocentrospora acerina*:

At 0°C: On both carrots there was bacteria and some chlamydospores on the tissue. On agar plates there was *M. acerina* and some unknown contaminations, including *Alternaria*-like fungus (not confirmed). On incubated tissue *M. acerina* spores and *Alternaria*-like mycelium were detected, and after splitting the carrot, the infection was going inside the core (1 cm for black dry symptoms, and 2 cm for black wet symptoms) as black lesions (Fig. 31B and E). Various re-isolations were attempted from different places, from the blackish to the healthy core, and there were always bacteria coming from this black lesion in the core of the carrot, while the fungus was isolated from the tissue between the symptomatic and healthy appearing areas (Fig. 31).

At 3°C: On both carrots chlamydospores and bacteria were found. When splitting the carrot tissue, the diseased tissue (blackish) went into the core about 1.5 cm for the black and dry symptoms and 2 cm for the black and wet symptoms. On culture media bacterial growth, white mycelium, black mycelium, and *Alternaria* spores were found. While on incubated tissues, *M. acerina* and *Alternaria*-like spores were found.

At 6°C: On both carrots both chlamydospores and bacteria were found, and when the carrot tissue were split, the infection was going into the core as blackish lesions, about 3 cm for the black, dry symptoms (Fig. 31B) and 1.5cm for the black, wet symptoms (Fig. 31E). In culture, bacteria, chlamydospores and some unknown contaminants were detected. While on the incubated tissue *M. acerina* was found sporulating. About 75% of the samples had dry and black symptoms at 0°C however this percentage decreased by increasing room temperature; 60 and 40% at 3 and 6°C respectively.

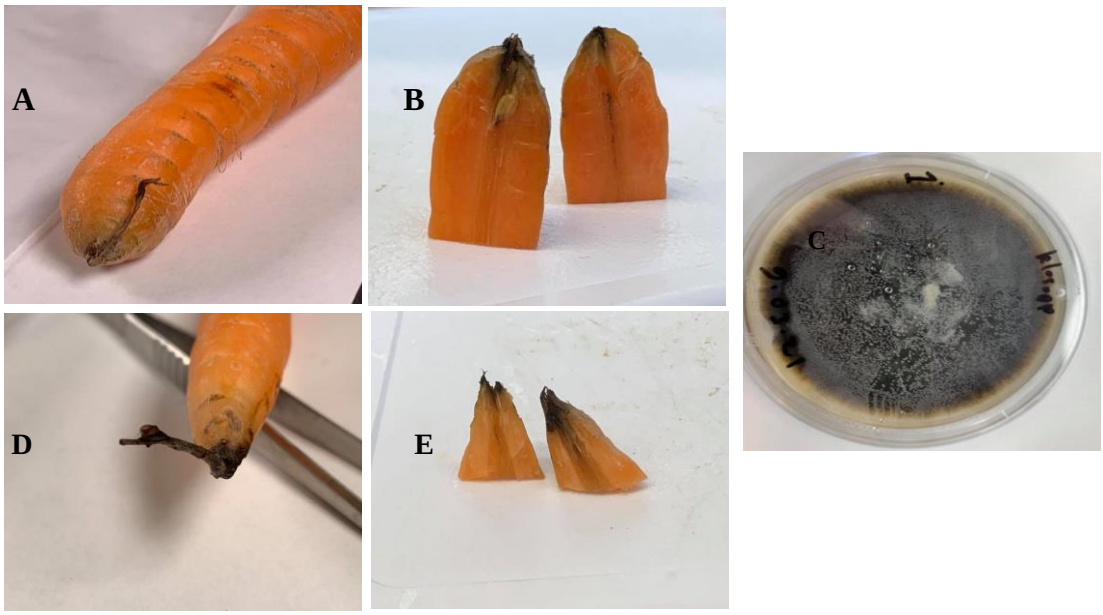

Fig.31. The dry and wet type symptom of *Mycocentrospora acerina*; dry type tip rot (**A**); the same carrot after splitting (**B**); wet type tip rot (**D**); and shows the same carrot after splitting (**E**). *M. acerina* on agar PDA media (**C**). Photo (R. Amin).

3.4.2. *Cylindrocarpon* spp.:

At 0°C: On carrot tissue we found *Cylindrocarpon* spp. spores and bacteria, and after splitting there was a dry rot in core area, becoming dark brown and going deep into the tissue about (2 mm). On incubated tissue there were *Cylindrocarpon* spp. and *Alternaria*-like spores, and on agar media we found mycelium of *Cylindrocarpon* spp. with spores and some contamination resembling *Verticillium.* At 3°C: On the carrot tissue we found whitish mycelium and after splitting the symptoms inside were like dry under the tip but not going deep about (2mm). On agar media there was only bacterial growth, while on incubated tissue we found *Cylindrocarpon* spp.

At 6°C: On both carrots, spores of *Cylindrocarpon* spp. and *Alternaria* were found. After splitting, dry lesions of about 2mm went into the tissue (core and flesh). On agar culture *Cylindrocarpon* spp. mycelium and spores were found in addition to bacteria. On incubated tissues it was *Cylindrocarpon* spp. and *Alternaria*-like spores were found (Fig. 32). The symptoms were getting darker at higher temperature.

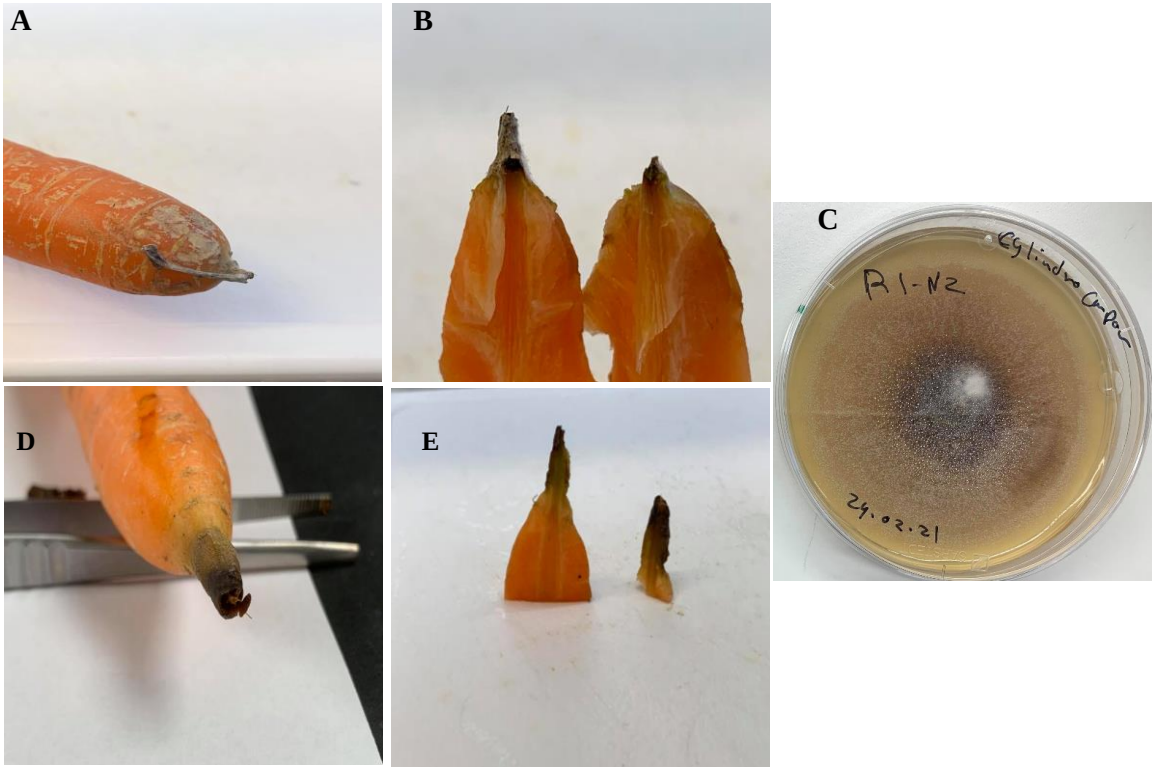

Fig.32. The dry and wet type symptom of *Cylindrocarpon* spp.; dry type tip rot (**A**); and the same carrot after splitting (**B**); wet type tip rot (**D**); and shows the same carrot after splitting (**E**). *Cylindrocarpon* spp. on agar PDA media (**C**). Photo (R. Amin).

3.4.3. *Dictyostelium* spp.:

At 0℃: On carrots tissue was found only whitish mycelium growing and after splitting there was a dry rot in core area, becoming dark and going deep into the tissue about (5mm). In addition, on incubated tissue there was *Dictyostelium* spp. spores and *Alternaria*- like spores (Fig. 33).

At 3℃: On the carrot tissue, it was found *Dictyostelium* spp. spores and whitish hyphal growth, and the same spores were found on agar media with unknow mycelium. The carrot did not split because the infection was only on the tiny taproot, whereas on incubated tissue showed up *Dictyostelium* spp. and *Alternaria*- like spores.

At 6℃: On carrot tissue was found some unknown spores and *Alternaria*-like, and after splitting there was a dry rot in core area going deep into the tissue about (5 mm). On agar culture and incubated tissue, was found bacteria, some unknow contaminant mycelium and *Dictyostelium* spp. spores. The symptoms were getting darker at higher temperature.

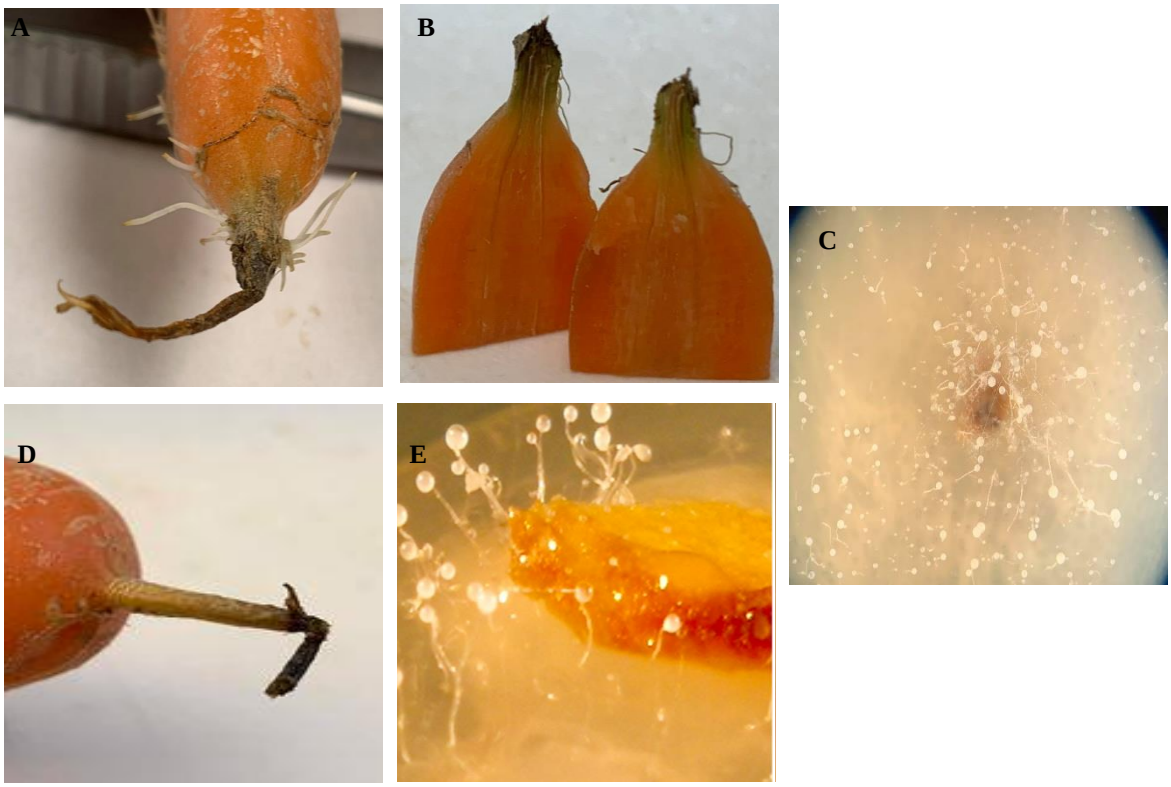

Fig.33. The dry and wet type symptom of *Dictyostelium* spp.; dry type tip rot (**A**); and the same carrot after splitting (**B**); wet type tip rot (**D**); and shows *Dictyostelium* spp. Hyphae and spores on tissue and agar (**E, C**). (Photo E by B. Asalf and the rest by R. Amin).

3.4.4. *Fusarium* spp.:

At 0°C: On carrot tissue some of *Fusarium* spp. spores showed up, and after splitting there was a dry rot in core are, becoming brown and going deep into the tissue about (20 mm) from the taproot. On agar culture it was found *Fusarium* spores and bacteria, while on incubated tissue was only *Fusarium* spp. spores appeared (Fig. 34).

At 3°C: On the carrot tissue was found *Fusarium* spp. spores, and after splitting there was a dry rot in core area, becoming brown. On agar media *Fusarium* spp. spores showed up, whilst on the incubated tissue were *Fusarium* spp. and *Alternaria*-like spores appeared.

At 6°C: On the carrot tissue was found bacteria and some *Fusarium* spp. spores, in addition it was a yellowish crystal slimy material on the tissue of the taproot. After splitting there was a dry rot in core area and going deep into the tissue about (5 mm). Whereas on agar culture and incubated tissue, showed up bacteria and *Fusarium* spp. (both mycelium and spores). The symptoms were getting darker at higher temperature.

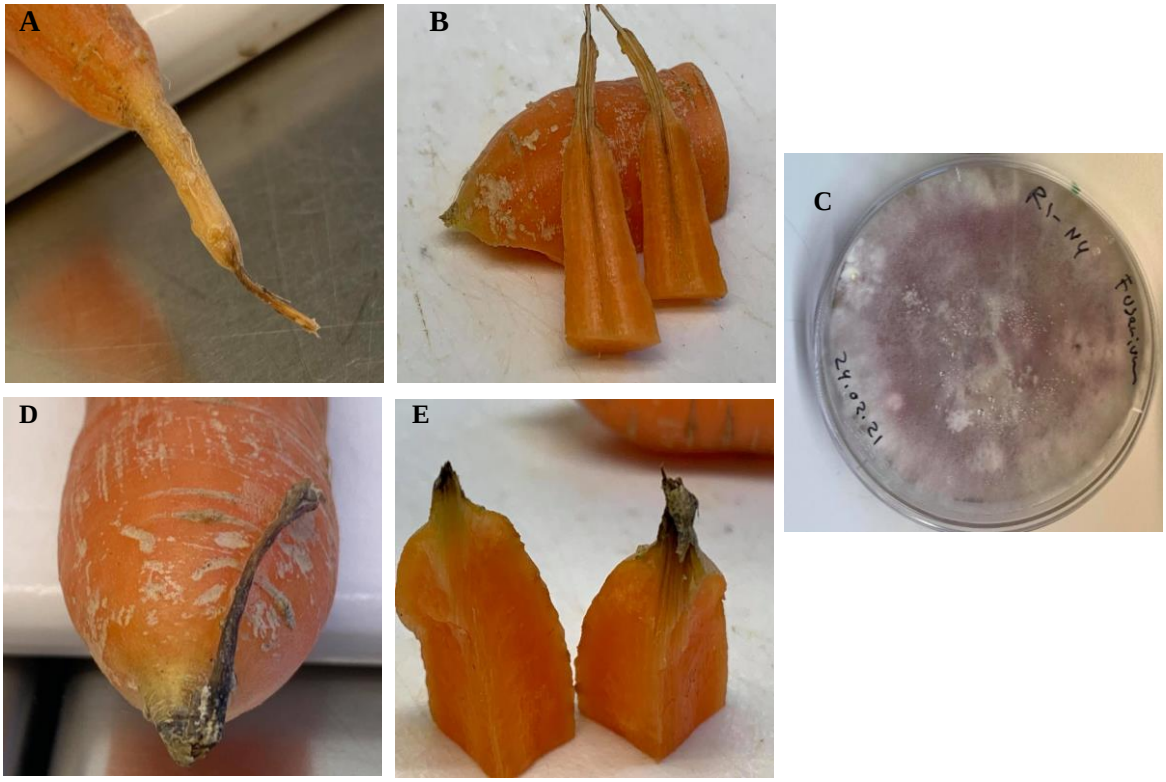

Fig.34. The dry and wet type symptom of *Fusarium* spp.; dry type tip rot (**A**); and the same carrot after splitting (**B**); wet type tip rot (**D**); and shows the same carrot after splitting (**E**). *Fusarium* spp. on agar PDA media (**C**). Photo (R. Amin).

4. Discussion:

We have fulfilled Koch's postulate and confirmed that tip rot may be caused by several pathogens. Tip rot is a disease complex of stored carrots caused by different fungi. At the early stage of infection, the symptom from all pathogens looks alike but as the disease progress and pathogen structures like chlamydospores formation become visible on the tissue, some of the pathogens for example *M. acerina* can be easily identified. The causal agents of tip rot can develop at very low temperature near to 0°C and all the four-carrot cultivar included in this study were susceptible to the candidate pathogens. Both disease development and length of the latent period from time of inoculation to first symptoms start to appear, were influenced by storage temperature and cultivars. The higher the storage temperature, the more rapid the tip rot developed and the shorter the latent period. When inoculated in the taproot, symptoms started as discoloration and necrosis from the inoculation point and progressed upwards. The lesions were either dry or wet. The wet type symptoms were mostly associated with secondary invaders like bacteria and other saprophytes.

For correct identification of tip rot, carrots have to be washed and be free from soil, the taproot has to be split and investigated for the extent of the lesion, where it occurred (in the core or the whole tissue colonized), tissue discoloration. Moreover, identification of the causal agent based on symptom was further complicated by secondary invaders like *Mucor*, *Rhizopus*, bacteria, coinfection of the carrot by multiple pathogens at the same time or sequentially and effect of long-term storage on carrot physiological, especially on the tip of the taproot. Since tip rot symptom development was affected by many factors, for correct identification of the causal agent(s) of tip rot, it is important to confirm the pathogen identity by microscopy or DNA sequencing.

Our results about tip rot symptoms agreed with the description of tip rot in the pre-project report (Nærstad, 2015). Among the fungi that cause tip rot are *Mycocentrospora acerina*, *Cylindrocarpon* spp., *Dictyostelium* spp. and *Fusarium* spp. Additionally, other fungi were found like; *Alternaria* spp., *Botrytis cinerea*, *Rhizoctonia* spp., *Mucor*, *Rhizopus,* and *phoma* spp. and they may be also play a role in tip rot development by themselves or interacting with the four identified fungi. The different symptoms of tested fungi were as follows:

1. *Mycocentrospora acerina* caused black symptoms which were almost associated with either wet or dry lesions, and the infection was like a black lesion going deep in core area. And that agrees with previous study results, which tip rot symptoms described as black infection in the tip of the taproot (Røeggen, 1973). However, lesion's structure (dry and wet) could be due to the other contaminates that were found on the lesions like *Alternaria* spp., bacteria, and production of chlamydospores of *M. acerina* in our results. In addition, the black lesion which goes deep in the core area was described as a kind of cell-wall polysaccharide degradation enzymes that *M. acerina* produces in vitro (Le Cam et al., 1997). **2.** *Cylindrocarpon* spp. caused brown to dark brown and black discoloration in the point of

inoculation. This result was agreed with pre-project report (Nærstad, 2015) where *Cylindrocarpon* spp. symptoms described as discoloration in the tip of the taproot. The variation of the discoloration from brown to dark brown and black could be due to the contaminants that were found on the tissue like *Alternaria* spp., bacteria, producing chlamydospores of *Cylindrocarpon* spp. and nematodes. However, the way that the dry and brown lesion went deep in core area and the flesh area surrounding the core, did not described in previous studies. **3.** *Dictyostelium* spp. showed dry light brown to dark brown symptoms and the variation in colour was due to the secondary contaminants like *Alternaria* spp. and bacteria. There were limited literatures about *Dictyostelium* spp. as a cold storage disease, and there is a need to do further studies based on our results about this pathogen in the future. **4.** *Fusarium* spp. caused dry symptoms on the point of inoculation and after splitting the carrot, a dry, light to dark brown lesion went deep in the core. Our results agreed with (Howard et al.,1996) in the description of *Fusarium* spp. symptoms on carrots which described it as a dry and crumble tip of taproot. However, the variation of the lesions between light and dark brown could be due to the secondary contaminants that were found like *Alternaria* spp. and bacteria.

Latent period and severity results showed that: *Mycocentrospora acerina* had the shortest latent period and the disease development was faster than the other pathogens. It could be an interaction between *M. acerina* and *Alternaria* spp. which made the disease more sever and developed faster, especially it was no interaction effect of temperature and cultivar (P = 0.198) on disease development (AUDPC). *Alternaria* spp. was predominant on the infected carrot lesion and especially at 6℃. In addition, the incidence varied between the cultivars, e.g. the cultivars (Romance, Dailyance, Brillyance and Namdal) which inoculated with *Cylindrocarpon* spp. at 6℃ on the date (25.11), the incidence was (100, 60, 80 and 100%).

The identification of the causal agent of tip rot is crucial to implement appropriate control measures because some pathogens may cause very similar symptoms, e.g. black spot diseases on carrots that appear postharvest may be caused by *A. radicina, A. dauci* and *Rhexocercosporidium carotae* (Voorrips et al.,2006). In addition, it could be different pathogens so same treatment will not affect the disease e.g., fungi and Oomycetes have different sensitivity to conventional fungicides due to their differences in biosynthesis pathways (Latijnhouwers et al.,2003). Therefore, to control any plant pathogen, the correct identification of the causal agent is crucial.

Many challenges were faced during this study, one of the challenges was to find the most realistic inoculation method, and many methods were tested on a pilot study and rejected due to their results, for instance: **1.** Inoculate carrot slices by using agar plug: In this method the severity of the infection was low, and the agar plug dried, and fungus could not penetrate carrot tissue although the infection incidence was 100%

2. Cutting the carrots from the mid and inoculate by agar plug: In this method the incidence of the infection was low, and many carrots formed callus on the taproot and fungus died especially the carrots were incubated at higher temperature with the presence of light.

3. Inoculate the taproot by spore suspension and agar plug: In this method, we found that splitting the end of the taproot was the best method to help holding the agar plug on the tip of the taproot and the incidence of the infection was 100% in all 20 carrots, while the incidence of the infection by spore suspension was about 80%. In addition, due to Covid-19 lockdown and isolation we had a long registration interval so, it was some missing data were not included in analysis. All these challenges constituted an obstacle to understand the causal agent(s) of tip rot of carrots, however we were able to identify the predominant pathogens and categories them based on their tip rot symptoms and that was through a hug job of morphological identification, isolation, metabarcoding, inoculation, and fulfilling Koch's postulates.

Although the early stage of tip rot identification by symptom was difficult, here I have proposed the following as guide for the identification of the four candidate pathogens based on the symptoms (black, dark brown, and light brown) and lesions texture (dry and wet) and growth of the pathogen inside the carrot (core, flesh) (Table. 3).

Table 3. Description of symptoms of the different pathogens on carrot tissue and after splitting the carrot with their lesion's structures.

Pathogens	Symptoms on tissue	Symptoms after splitting the carrot	Lesion's structure
Mycocentrospora acerina	Black infection starts from the tip of taproot.	The infection goes inside the carrots in the core as a black discoloration, or black material.	Dry and wet.
Cylindrocarpon spp.	Brown, dark brown to black.	Drying the whole area (core with surrounded flesh) and getting brown to black.	Dry and wet.
Dictyostelium spp.	Light brown, brown to dark brown.	Dry core and brown, infection go deep in the core about (2mm).	Dry and wet.
Fusarium spp.	Light brown, brown and sometimes get pink.	Dry core and brown, infection deep (5-20 mm).	Dry (typically) but can be wet too.

4.1. Conclusion:

Tip rot of carrot is caused by biotic agents and is a disease complex of stored carrots. Many fungi cause tip rot of carrots, the most abundant identified in this study were *Mycocentrospora acerina, Cylindrocarpon* spp., *Dictyostelium* spp., and *Fusarium* spp. Many factors affect typical tip rot development such as temperature, secondary invaders, and root physiology due to long term storage, so tip rot causal agent identification based on symptom on the tip of the carrot is difficult especially at the initial stage of infection. To correctly identify the causal agent, one has to split the carrot and see how deep the symptom goes in the taproot and check the pathogen structure as spores and chlamydospores, hyphal structure under a microscope or do DNA based identification.

4.2. Recommendations:

I have few points that I would like to recommend for the future studies on tip rot of carrots:

1.The results at 0℃ showed that the infection goes deep in the core for all candidates including *Dictyostelium* spp., while at 3 and 6℃ the infection was not going deep in the core for this pathogen, even though the pathogen was not growing too much. And I think the symptoms that all candidates showed at 0℃ were remarkably and should be studied further because as it is known the carrots store at 0℃ after harvesting for several months.

2.I would like to recommend including more pathogens such like: *Alternaria* spp., *Rhizoctonia* spp., *Botrytis cinerea, Phoma* spp. in the future testing, because those pathogens were founded on the tissue of carrots during identification process. And especially *Alternaria* spp. because it forms chlamydospores same as *M. acerina.* and it was found on about all isolates, and predominant on the isolates that inoculated with *M. acerina* and stored at 6℃.

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Appendix:

Fig.35. Washing carrots (**A**); and sorting carrots (healthy and with tip rot symptoms) (**B, C**) Photo (R. Amin).

Fig.36. Taking samples for DNA extraction from healthy ones (**A**); isolating samples with tip rot symptoms (**B**). Photo (R. Amin).

Fig.37. DNA extraction procedures (**A**); making agar plates PGA (**B**); making agar plates PDA (**C**); sowing 4 cultivars of carrots in pots to produce healthy carrots (**D**). Photo (R. Amin).

Fig. 38. Producing spores of the candidates by inoculating fresh carrot leaves to use them in different inoculation methods (**A, B**). Photo (R. Amin).

Fig.39. Trying different inoculation methods to figure out the optimal ones based on their results; inoculating near of the crown with different candidates (**A**); inoculating the carrots with *Fusarium* spp. and the other candidates by making a wound in the middle of carrots (**B**); inoculating the carrots by cutting them from the middle and placing an agar plug on the tip of taproot (**C**); cutting the tip of taproot with about 2cm of the base and placing an agar plug of different candidates (**D**); cutting each carrot to many slices (about 2cm) and placing an agar plug of each candidates (**E**); inoculating the carrots with *Mycocentrospora acerina* and the other candidates by making a wound in the taproot and using both agar plug and in another boxes spore suspension was used (**F**). Photo (R. Amin).

Fig.40. Harvesting the carrots (A); and store them at 0℃ until inoculation time (B). Photo of pure inoculum of the candidates: *Dictyostelium* spp., *Fusarium* spp., *Cylindrocarpon* spp. and *Mycocentrospora acerina* respectively with both sides (C, D). Photo (B. Asalf).

Fig.41. Inoculating procedures; preparing inoculation boxes (**A**); surface sterilizing the carrots (**B**); control boxes "untreated" (**C**); splitting the taproot of the carrots (**D**); control box "pure agar plug placed on the taproot" (**E**); placing an agar plug (5mm) on the taproot for each pathogen (**F**). Photo (B. Asalf).

Fig.42. Inoculated carrot boxes (two replicates of each pathogen) at each room temperature; boxes at 0℃, and one logger hang in the room to check the humidity in the room by the time (**A**); boxes at 3℃(**B**); boxes at 6℃ (**C**); control box with logger inside it at 3℃ and we had a logger inside the control boxes at the 0℃ and 6℃ too to check the humidity and temperature inside the boxes by the time (**D**). Photo (R. Amin).

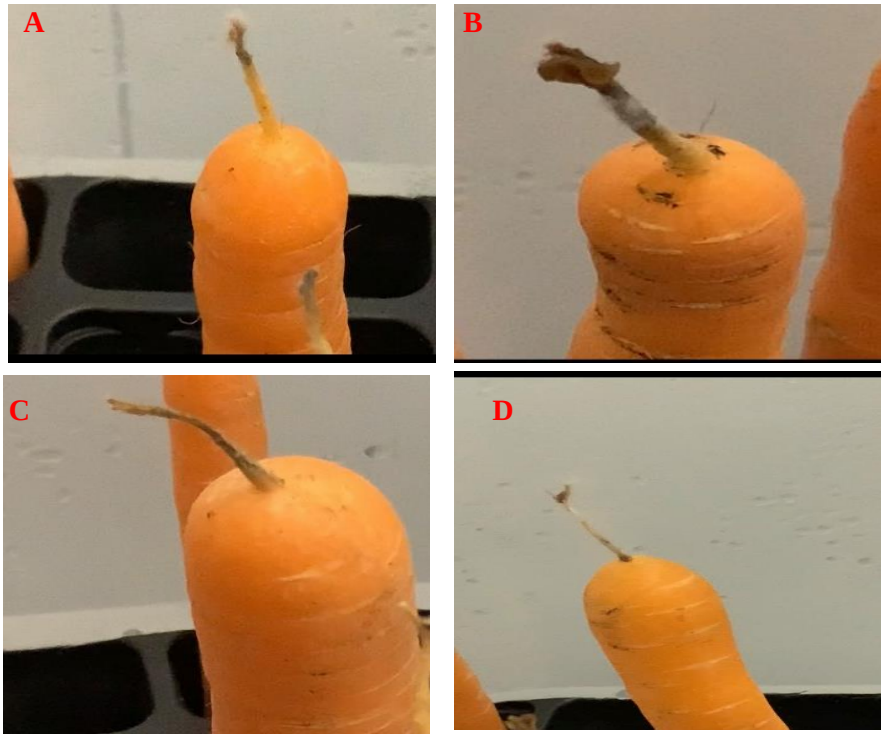

Fig.43. Examples of the infection size during registrations; infection size 5mm (**A**); infection size 15mm (**B**); infection size 17.5mm (**C**); infection size 20mm (**D**). Photo (R. Amin).

Fig.44. Different pathogens at each room temperature; *M. acerina* at 0°C, 3°C, 6°C respectively (**A, B, C**); *C. destructans* at 0°C, 3°C, 6°C respectively (**D, E, F**); *D. discoideum* at 0°C, 3°C, 6°C respectively (**J, H, I**); *F. avenaceum* at 0°C, 3°C, 6°C respectively (**J, K, L**). (Photo D, E and L by B. Asalf and the rest by R. Amin).

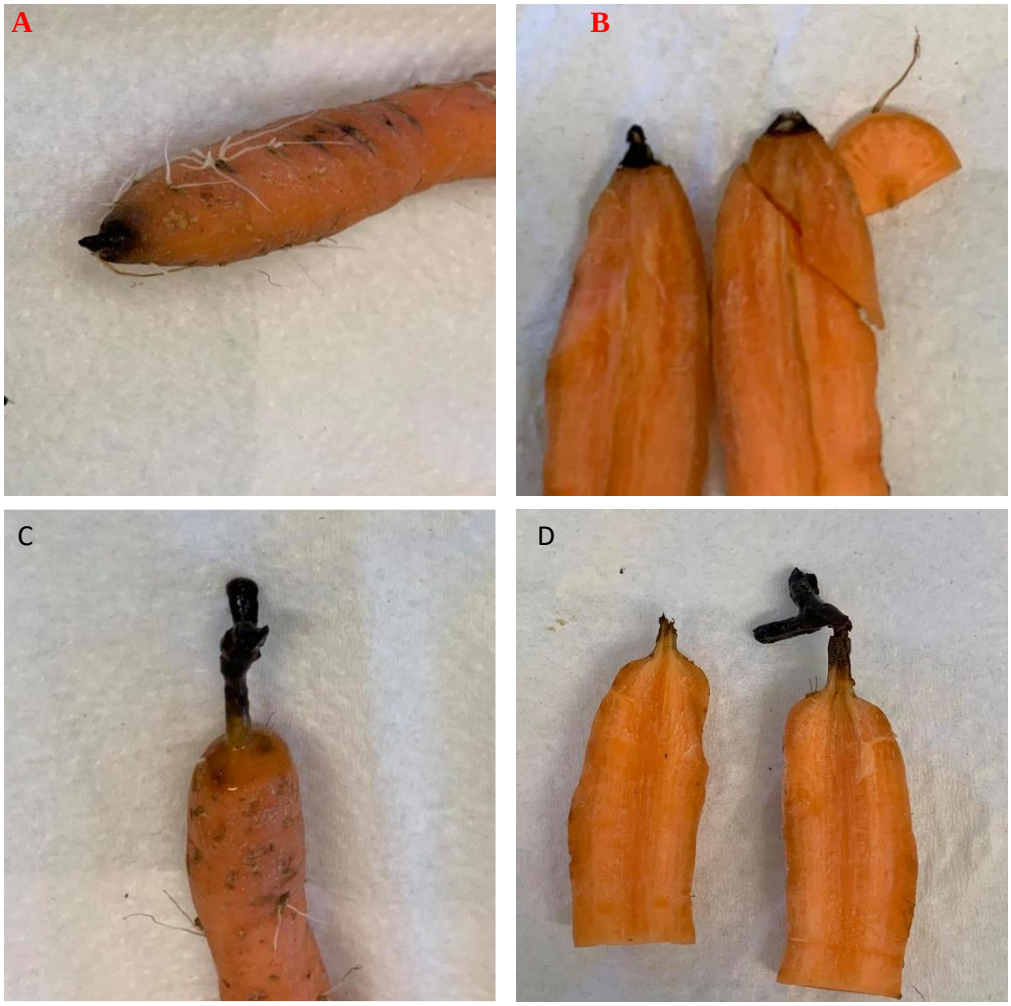

Fig.45. Inoculated carrots with *Alternaria*-spores that re-isolated from the inoculated carrots with *M. acerina*; dark black symptoms on tissue and after splitting it (**A, B**); dark black and wet symptoms on tissue and after splitting it (**C, D**). Photo (R. Amin).

Norges miljø- og biovitenskapelig universitet
Noregs miljø- og biovitskapelege universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway