Full-length article

Airborne basidiospores as an inoculum source of *Typhula variabilis* and the effect of hilling on the incidence of Typhula winter rot of carrots

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Abstract

Typhula winter rot on overwintering carrots caused by *Typhula variabilis* is a newly confirmed disease, and no practical control measure is yet available. To develop a control method, here we researched the infection period of *T. variabilis* and the time that winter rot appeared on carrots. Using spore traps, we found that basidiospore rain occurred from September to November before snowfall in Memuro, Hokkaido. In addition, carrot leaves collected in autumn had already been infected by *T. variabilis.* These epidemiological investigations revealed that the pathogen releases basidiospores to infect carrot leaves before snow cover, resulting in root decay under snow. An effective control method was then developed to avoid direct contact of *T. variabilis* basidiospores with plant tops by covering the plants with soil in autumn. Thus, the percentage of rotted roots was reduced to about half.

Keywords cultural control, infection period, overwintering carrots, spore dispersal, *Typhula variabilis*

Introduction

Typhula variabilis Riess has recently been recognized as a pathogen that causes Typhula winter rot of overwintering dicots under snow, especially carrots (Ikeda et al. 2016). *Typhula ishikariensis* Imai, a soilborne pathogen of the same genus, also causes damage to carrots (S. Ikeda, personal observation), as well as monocots such as winter wheat and turfgrass. Although the disease on the latter two crops may be controlled by spraying fungicide, no control measure is available for carrots. In addition, little is known about the epidemiology of *T. variabilis*.

We first considered that *T. variabilis* may infect carrots under the snow, as in the case with *T. ishikariensis* on monocots (Oshiman 1999). However, our previous study showed that the monokaryon of *T. variabilis* is pathogenic to carrots (Ikeda et al. 2016), and we found that *T. variabilis* releases basidiospores for 2 months, starting from September until snow falls (end of November and December in Hokkaido). On the other hand, the monokaryon of *T. incarnata* is weakly pathogenic (Matsumoto 1989) and may infect winter wheat before the snow season (Matsumoto et al. 1982). Such circumstantial evidence suggested that basidiospores of *T. variabilis* were the primary source of inoculum on overwintering carrots that remain in the field. Therefore, we studied the basidiospore dispersal period and the infection period. In addition, we report the results of field experiments showing that hilling to cover carrot plants with soil in mid-October is an effective means of decreasing the incidence of winter rot of carrots caused by *T. variabilis*.

Materials and methods

Spore trap

For specific detection of airborne basidiopores, we followed the technique described by Adams et al. (1984) using monokaryon testers of *T. variabilis*. Four monokaryons of *Typhula variabilis*, isolates S3e1, S3e2, S3e5, and S3e7 (MAFF 244294 to 244297, respectively; Ikeda et al. 2015) stored in a refrigerator at 0°C were used for the specific detection of spore rain of the fungus. Testers were grown for 14 days at 10°C in Falcon multiwell plates (12 wells/plate; wells 2.2 mm across and 6.0 mL in volume). Three wells each filled with 3.5 mL potato dextrose agar (PDA, Difco, Detroit, MI, USA) were inoculated with the four testers in one plate. Three plates each were placed on the ground at 1-week intervals in two locations 150 m distant from each other from September to November 2011 in Memuro, Hokkaido (42°53′29″N, 143°4′42″E, soil type: Light-colored Andosol. Our field experiments described below were put in the same location). One location was bare ground in a carrot field after harvesting, and the other was in an oil seed canola field where canola residues were left. In 2012, we put three plates each on and 1.8 m above the bare ground in the same area of the oil seed canola field. Lids of the plates were removed for 24 h to expose tester colonies to spore rain. After exposure, the plates were incubated at 10°C for 2 weeks, and subcultures from each well were examined for the presence or absence of clamp connections. Dikaryotization with the testers indicated the capture of *T. variabilis* basidiospores.

Disease development on carrots

Twenty carrot leaves (cvs. Koyo-nigo and Trophy) were randomly collected from the field in Memuro every week from September to November in 2011 and 2012. Each leaf was incubated at 4°C for 3 months in a plastic bag (test A). Each bag was examined for the presence of dark, small (1–2 mm in diameter) sclerotia. If present,

five sclerotia each were collected from the bag to isolate the fungus. The isolates were mated with tester monokaryons of *T. variabilis* S3 to confirm the identity.

In 2012, carrot leaves were washed in running water for 4 h and cut into 2-mm^2 fragments, sterilized in 70% ethanol for 3 min and in 1% (as active chlorine) sodium hypochlorite for 1 min, rinsed in sterilized distilled water three times, and then incubated on PDA plates at 4°C for 3 weeks. About 100 fragments were used each time. Colonies growing on the plates were identified as *T. variabilis* by the di-mon mating test. Finally, the residue of the leaves were put in a plastic bag to maintain humidity and incubated at 4°C for 3 months (test B).

Twenty carrot roots were collected from the field in Memuro at 10-day intervals from November 30 to March 20 in the winter of 2011/2012, and 30 to 50 roots were collected at 10-day intervals from December 30 to March 9 in the 2012/2013 winter. Carrot roots were excavated and washed to remove soil and kept in plastic bags at 4°C for 3 months. Small, dark sclerotia on rotten roots were reisolated as signs of *T. variabilis* infection.

In 2010 and 2011, carrots (cvs. Koyo-nigo and Trophy) were sown in the field in Memuro in June, and the soil was ridged to cover the plants, using machinery for potato culture, in mid-October. Carrots were dug from an area of 1.5 × 1.0 m the following spring. The percentage of rotten roots was determined, and the presence of sclerotia was surveyed. Three replicates were used in April 2011 and four replicates in April 2012. Carrots grown in a level row were used as the control.

Results

Spore dispersal

Typhula variabilis basidiospores were trapped from September through November in both 2011 and 2012 (Fig. 1). Spore rain occurred regardless of the presence or absence of plants in the field in 2011, and traps placed both on and above the ground showed the same pattern of spore dispersal in 2012. *Typhula variabilis* could not be detected from traps placed on September 11 or 18 in 2012 because trap plates were badly contaminated with various fungi, including species of *Fusarium*, *Alternaria*, and *Rhizopus*, and the data were excluded. Although plates set on November 13, 2012 were contaminated by bacteria due to precipitation during the night, a total of 10 wells in six plates captured *T. variabilis*. The fungus was trapped in a single well set on the ground and in nine wells installed 1.8 m above the ground.

The daily temperature range fluctuated between 0 and 25°C when spore rain occurred (Fig. 1). Spore dispersal culminated when the mean temperature was around 10°C, with a maximum around 20°C and a minimum between about 0 and 5°C. Spore rain stopped when mean temperature decreased below 1°C, with minimum and maximum temperatures below about 0 and 5°C, respectively.

Infection period of *Typhula variabilis* on carrot leaves

Dark sclerotia, 1–2 mm in diameter, were formed after a 3-month incubation on carrot leaves collected from the field from September to November in both 2011 and 2012, with the exceptions of leaves collected on September 20 and November 8, 2011 (Table 1, test A). Fungal isolates from the sclerotia were all identified as *T. variabilis* by mating tests with testers of the fungus. Washing the leaves in running water for 4 h failed to remove the fungus; *T. variabilis* sclerotia were formed on the leaves (Table 1, test B). Despite 100 leaf segments being examined each time, direct isolation from the leaves was always unsuccessful.

Carrot root rot under snow

In the field, dark sclerotia were present on leaves in late January, but carrot roots were not rotten until the end of the experiments in March in both winters (Table 2). Samples retrieved from the field and incubated in plastic bags were invariably found to be rotten to variable extents, with dark, small sclerotia on the surface. Sclerotia of other *Typhula* species were not found.

Cultural control of Typhula winter rot

Covering carrot plants with soil in mid-October significantly reduced the disease incidence in both cultivars and both years (Fig. 2). No root rot was observed in Trophy in 2011, whereas in 2012 it showed an incidence rate similar to that of Koyo-nigo.

Discussion

Basidiospores are not generally the major inoculum source in the genus *Typhula*, except in *T. incarnata* Lasch. Our findings show that *T. variabilis* is an airborne pathogen, initiating infection on carrot leaves, even though direct isolation from leaves was unsuccessful. The leaves collected in autumn ultimately produced sclerotia when incubated in plastic bags, and the roots retrieved from the field also rotted when incubated in plastic bags. These results highlight the epidemiological difference from another winter pathogen, *T. ishikariensis* biotype A, and suggest a unique control measure.

With regard to the airborne epidemiology of *T. variabilis*, the fungus first infects carrot leaves and crowns exposed to the air via basidiospores and then invades crowns to cause root rot under the snow. Consequently, hilling of carrots with soil in

mid-October resulted in successful disease control by preventing infection of the leaves. Hilling was done with machinery for potato culture, and carrot crowns were buried at 5 cm below the surface of the ground. Carrot leafstalks are about 15 to 30 cm long, and leaves grow thick on the upper half side of the stalks generally. In our experiments, all carrot leaves were exposed to sunlight until snow cover. Therefore, our hilling treatment does not affect photosynthesis. In fact, free amino acid concentration increased in overwintering carrots cultured with hilling, and the taste was confirmed as palatable (Otsuka 2014). On the other hand, hilling treatment in September is expected to be more effective to inhibit winter rot because spore dispersal culminates in mid-October. Nevertheless, we do not have any information about quality in the case of hilling in September. In addition, although higher hilling is expected to better suppress the disease, harvesting in the spring will be very difficult for.

Typhula winter rot on carrots is caused by *T. variabilis* in Memuro, where the pathogen *Typhula japonica* Terui seldom occurs. However, *T. japonica* often occurs on carrots in Sorachi and Okhotsk. *Typhula japonica* also seems to infect carrots through basidiospores, which are normally dikaryotic and agglutinate in a mass on the basidiocarp (Ikeda et al. 2015). The infection strategy of *T. japonica* should be further investigated to establish whether this hilling countermeasure would also be successful against *T. japonica*.

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References

Adams TJH, Williams END, Todd NK (1984) A species-specific method of analyzing populations of basidiospores. Trans British Mycol Soc 82:359–361

Ikeda S, Hoshino T, Matsumoto N, Kondo N (2015) Taxonomic reappraisal of *Typhula variabilis*, *Typhula laschii*, *Typhula intermedia*, and *Typhula japonica*. Mycoscience 56:549–559

Ikeda S, Hoshino T, Matsumoto N, Kondo N (2016) Rot diseases of carrot and rapeseed caused by *Typhula* species under snow in Hokkaido, Japan. J Gen Plant Pathol 82:286–291

Matsumoto N, Araki T (1982) Field observation of snow mold pathogens of grasses under snow cover in Sapporo. Res Bull Hokkaido Natl Agric Exp Stn 135:1–10

Matsumoto N (1989) Autecology of the pathogenic species of *Typhula*. Res Bull Hokkaido Natl Agric Exp Stn 152:91–162

Oshiman K (1999) Change in isolation frequency of *Typhula ishikariensis* from turfgrass under snow cover on golf courses. Mycoscience 40:373–375

Otsuka S (2014) Quality property and cultural method on overwintering carrots with the aid of the weather condition in Hokkaido. Soil cultivation and ecological agriculture 47:7–10

Figure legends

Fig. 1 Occurrence of spore rain of *Typhula variabilis* (top) and ambient temperature changes (bottom) in 2011 and 2012. Falcon plates with 12 wells containing cultures of tester monokaryons were used to capture basidiospores. Monokaryon testers were dikaryotized when basidiospores were trapped in the well. A total of 36 wells were used each time. All but one of the wells on the ground was contaminated with bacteria on November 13, 2012; nine wells set 1.8 m above the ground were uncontaminated.

Fig. 2 The effect of hilling on the incidence of root rot in the two carrot cultivars. ** Significant difference ($P < 0.01$) in percentage of root rot between hilling treatments in each cultivar.

Table 1 Presence or absence of sclerotia of *Typhula variabilis* on carrot leaves after 3-month incubation at 4°C (test A) and after washing for 4 h under running water followed by a 3-month incubation at 4°C (test B)

Sampling date			Test A	Test B
2011	Sept.	20	-	
		27	+	
	Oct.	4	+	
		11	+	
		18	+	
		26	+	
	Nov.	1	+	
		8	-	
		15	+	
		22	+	
		30	+	
2012	Sept.	25	+	+
	Oct.	2	+	+
		9	+	+
		15	+	+
		22	+	+
		30	+	+
	Nov.	6	+	+
		13	+	+
		20	+	+

+: sclerotia present; -: sclerotia absent

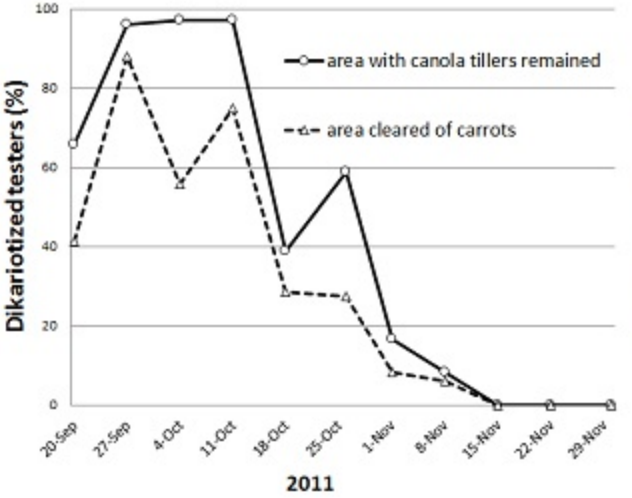
Only leaves that looked intact were collected. Mycelia developed from sclerotia were all identified as *T. variabilis* based on the di-mon mating test.

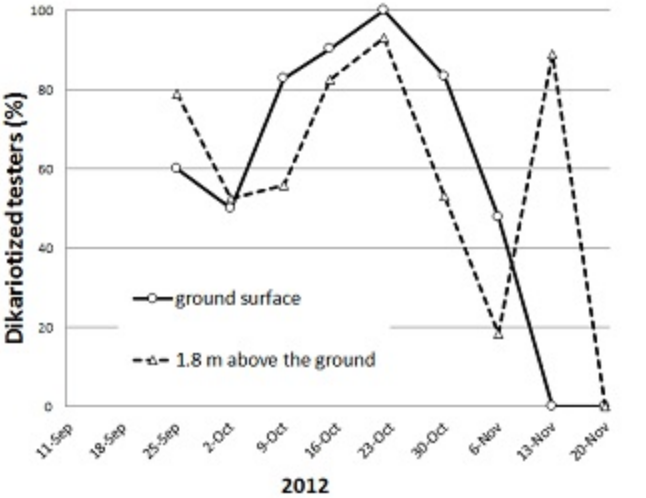
Table 2 Incidence rate of carrot root rot under snow

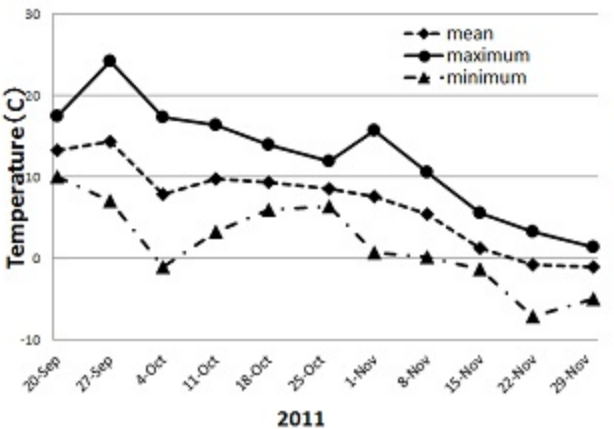
Sampling date			% rotted roots in the field[a]	% rotted roots incubated in plastic bags[b]
2011	Nov.	30	0	5.0
	Dec.	10	0	30.0
		20	0	10.0
		30	0	20.0
2012	Jan.	10	0	25.0
		20	0	65.0
		30	0	40.0
	Feb.	10	0	35.0
		20	0	35.0
	Mar.	1	0	30.0
		10	0	35.0
		20	15	55.0
2012	Dec.	30	0	27.1
2013	Jan.	9	0	16.0
		19	0	17.9
		30	0	7.6
	Feb.	9	0	15.2
		19	0	15.4
	Mar.	1	0	10.0
		9	3	43.1

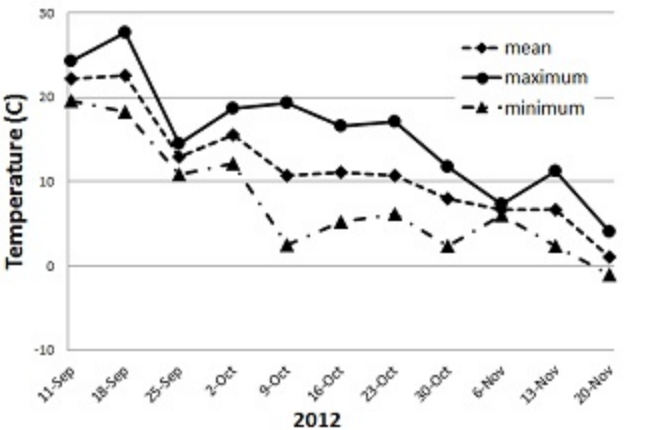
[a] Twenty roots were examined on each sampling date in the winter of 2011/2012 and about 30 to 50 in the winter of 2012/2013.

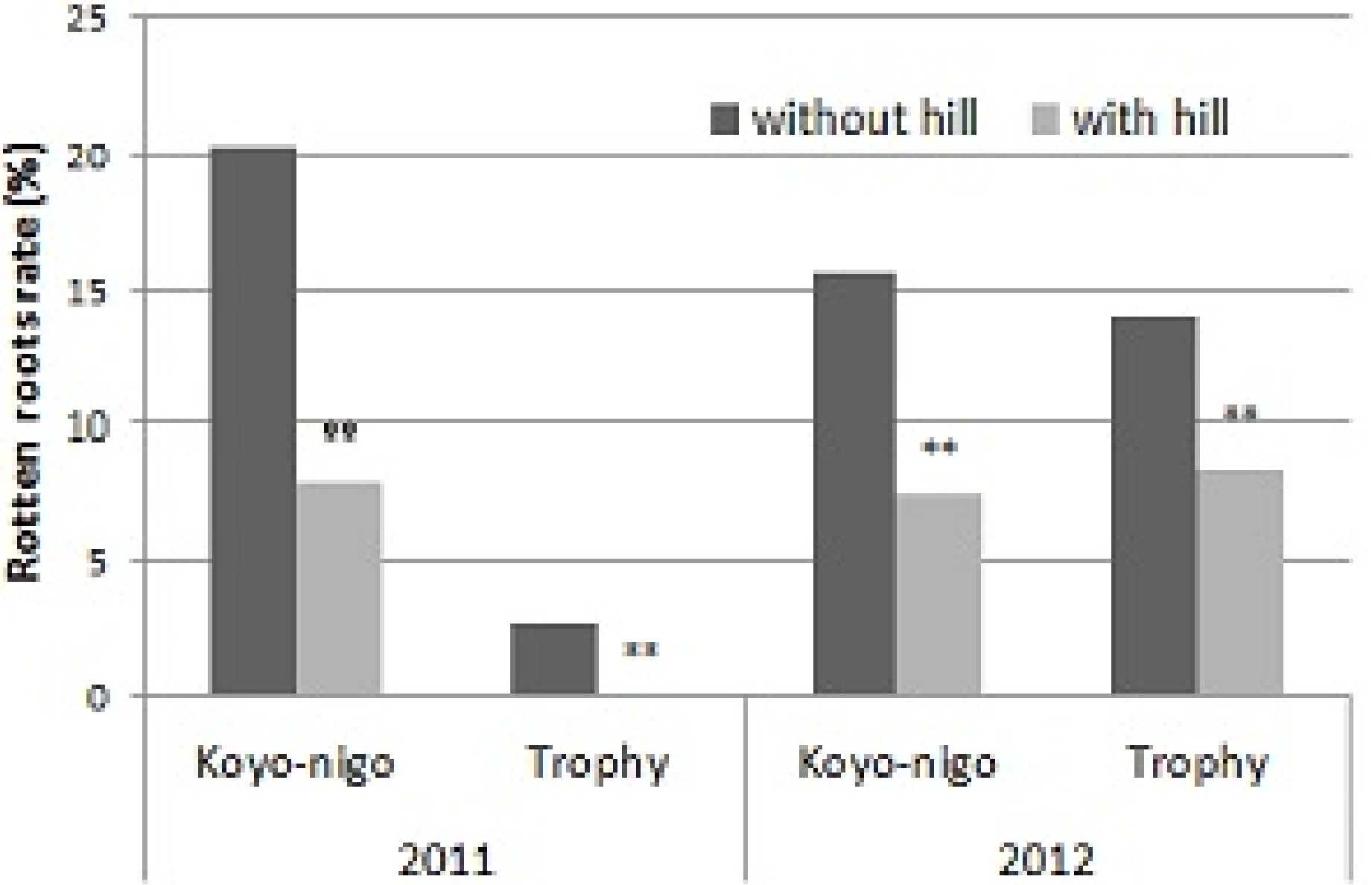
[b] Those roots that appeared intact after checking the rot rate (data in left column) were incubated in plastic bags at 4°C for 3 months.


Dikariotized testers (%)
area with canola tillers remained
area cleared of carrots
100
80
60
40
20
0
20-Sep
27-Sep
4-Oct
11-Oct
18-Oct
25-Oct
1-Nov
8-Nov
15-Nov
22-Nov
29-Nov
2011


Dikariotized testers (%)
ground surface
1.8 m above the ground
100
80
60
40
20
0
11-Sep
18-Sep
25-Sep
2-Oct
9-Oct
16-Oct
23-Oct
30-Oct
6-Nov
13-Nov
20-Nov
2012


Temperature (C)
mean
maximum
minimum
30
20
10
0
-10
20-Sep
27-Sep
4-Oct
11-Oct
18-Oct
25-Oct
1-Nov
8-Nov
15-Nov
22-Nov
29-Nov
2011


Temperature (C)
mean
maximum
minimum
30
20
10
0
-10
11-Sep
18-Sep
25-Sep
2-Oct
9-Oct
16-Oct
23-Oct
30-Oct
6-Nov
13-Nov
20-Nov
2012


25
20
15
10
5
0
Rotten rootsrate (%)
without hill
with hill
**
**
**
**
Koyo-nigo
Trophy
Koyo-nigo
Trophy
2011
2012