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In vitro therapies for virus elimination of potato-valuable germplasm in Norway

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ABSTRACT

Potato (*Solanum tuberosum* L.) is one of the most important crops grown in Norway, and virus-free plants are required for commercial potato production and for preservation of potato germplasm. The present study evaluates three *in vitro* therapies – meristem culture, cryotherapy, and chemotherapy combined with thermotherapy – to eliminate viruses from eight historically valuable potato cultivars belonging to the Norwegian potato germplasm. Potato virus Y, potato virus M, potato virus X and potato virus S were present in eight selected old potato cultivars due to long-term conservation in open field. Double-antibody sandwich enzyme-linked immunological assay (DAS-ELISA) and biological indicators were the standard tests used to confirm virus infection in our study. Six virus-free plants from four potato cultivars were obtained after meristem culture, and no virus-free potato cultivars were obtained after cryotherapy. Virus-free frequency for eight different potato cultivars after combining chemotherapy with thermotherapy varied from 36.4% to 100%, with single virus elimination rates of between 74.2% and 92.9%. Chemotherapy compared with thermotherapy was the most effective of the three *in vitro* therapies used in this study. Highly sensitive small RNA high-throughput sequencing (HTS) was used to evaluate the virus status of potato virus-free materials after virus eradication, and no virus was found, which was consistent with the results of DAS-ELISA and biological indicators. Small RNA HTS has been reported for the first time to evaluate the virus status after virus elimination and to control virus-free potato nuclear stocks.

1. Introduction

In Norway, potato (*Solanum tuberosum* L.) has a cultivation history of almost 250 years. Around 120,000 ha of potatoes are currently grown annually, with a total yield of 314,000 tons at a wholesale value of approximately NOK 500 million (Statistics Norway, 2017). Approximately 30% of the whole potato production is used for food consumption, 11% for seed potatoes, 18% for production of starch and alcohol, and the remaining proportion as animal feed and for use in private gardens (Naerstad et al., 2012). The average fresh consumption of potato is approximately 25 kg per person in 2017 (Development in Norwegian Diet 2017, Norwegian Health Department Report IS-2680).

The breeding of novel cultivars with attractive qualities for growers is constantly in demand in the market. This in turn implies the need for a reliable diverse germplasm collection (with the appropriate phytosanitary conditions and easy accessibility) for subsequent genetic improvements by means of classical and biotechnological breeding strategies. In Norway, a private open field collection of 170 potato cultivars collected by Erling Olsen has been evaluated by the Norwegian Genetic Resource Centre as 'high valuable' since it contains many historical cultivars. This led to a priority list of virus-free seed potatoes of selected cultivars from this collection, and 22 valuable accessions have been introduced into *in vitro* culture for conservation at the Norwegian Potato Gene Bank since 2015. Due to their long preservation time in open field, these collections were heavily infected with potato virus, especially potato virus S (PVS) and potato virus X (PVX). Virus-free seed potatoes are a pre-requisite to enhancing further use of these cultivars. Preservation of virus-free plant germplasm is necessary in exchanges of

Abbreviations: DAS-ELISA, double-antibody sandwich enzyme-linked immunological assay; GA_3, gibberellic acid 3; IBA, indole 3-butyric acid; LN, liquid nitrogen; LP, leaf primordia; MCM, meristem culture medium; MS, Murashige and Skoog; HTS, high-throughput sequencing; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PLRV, potato leaf roll virus; PVA, potato virus A; PVM, potato virus M; PVS, potato virus S; PVX, potato virus X; PVY, potato virus Y; PVS2, plant vitrification solution 2; RBDV, raspberry bushy dwarf virus; RIN, RNA integrity number; SMM, shoot maintenance medium; TomRSV, tomato ringspot virus; TSV, tobacco streak virus

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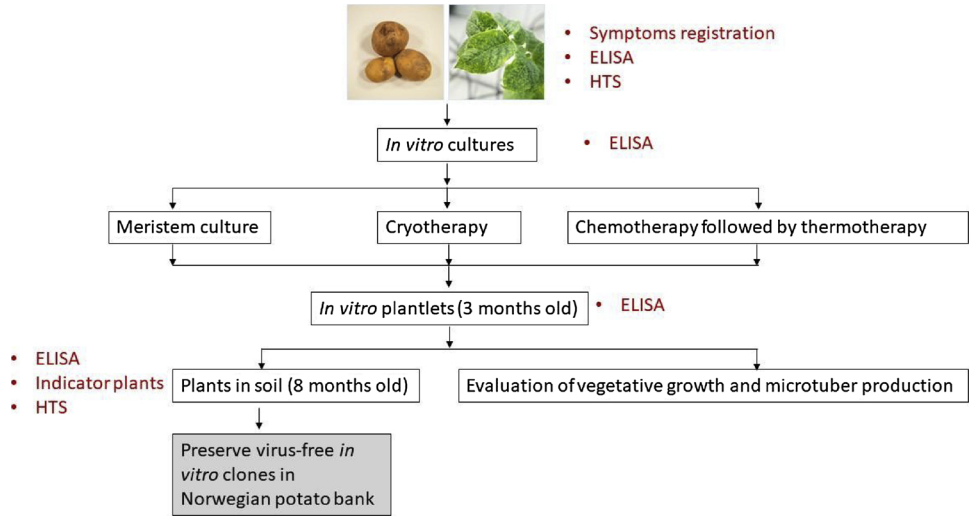

Fig. 1. A flow chart of *in vitro* therapies of potato and virus detection strategy.

plant materials between countries and in the use of preserved germplasm for breeding purposes (Wang and Valkonen, 2009; Wang et al., 2009).

In the present study, meristem culture, cryotherapy, and chemotherapy combined with thermotherapy were tested for virus elimination from eight valuable old potato cultivars in Norway. High-throughput sequencing (HTS), also called next-generation sequencing or deep sequencing, was used to evaluate the virus status of potato virus-free materials analyzed by enzyme-linked immunosorbent assay (ELISA) and biological indicators after virus elimination. The results provide a technical platform for establishing virus-free potato germplasm in Norway.

2. Materials and methods

A general flow chart of experimental procedures used in this study is presented in Fig. 1.

2.1. Plant materials

Eight potato cultivars ['Truls', 'Kerrs Pink blått skall', 'Gammelraude', 'Abundance', 'Gjernespotet', 'Hroar Dege', 'Iverpotet/Smaragd' and 'Sverre' (Fig. 2)] from Erling Olsen's collection were used in this study. All the potato cultivars were received as tubers (Fig. 2A, C, E, G, I, K, M and O) and grown in an aphid-free greenhouse. Once the plants reached 30–50 cm in height, they were evaluated for symptoms on fully opened leaves. Virus infection was tested by double-sandwich ELISA (DAS-ELISA; see below).

2.2. Establishment of diseased *in vitro* stock cultures

Single nodal segments (0.5–1.0 cm) were excised from potato plants grown in greenhouses and surface-disinfected with 75% ethanol for 1 min, 5% Deconex (VWR International AB, Stockholm, Sweden) for 10 min, and then rinsed thoroughly three times with sterile water. Shoot tips (2.0 mm in length) were excised from the disinfected segments and cultured on a shoot maintenance medium (SMM) for establishment of *in vitro* stock cultures. SMM contains Murashige and Skoog (1962) medium (MS) supplemented with 30 g/l sucrose, 1 g/l casein, 0.5 mg/l indole 3-butyric acid (IBA) and 9 g/l Bacto agar. The pH of the medium was adjusted to 5.8 prior to autoclave at 121° C for 20 min. *In vitro* cultures were maintained at a constant temperature of 20 ± 2 °C under a 16-h photoperiod with a light intensity of 50 μmol s^{-1} m^{-2} provided by cool-white fluorescent tubes. Subculture was done every three weeks.

2.3. *In vitro* therapies for virus elimination

2.3.1. Meristem culture

Meristems culture were performed according to Wang et al. (2006) with some modifications. The excised meristems were placed in culture tubes (20 mm x 150 mm) containing 5.5 ml of liquid meristem culture medium (MCM, pH 5.8) with a sterile autoclaved filter paper on the surface. MCM composed of MS medium supplemented with 20 g/l sucrose, 1 g/l casein, 0.1 mg/l IBA, 1 mg/l gibberellic acid 3 (GA_3) and 40 mg/l adenine hemisulphate. The cultures were kept in the same culture conditions as those used for stock cultures. Subculture was done every three weeks and twice on the same MCM medium before transferring to solid SMM.

2.3.2. Shoot-tip cryotherapy

Shoot-tip cryotherapy by droplet vitrification was conducted according to Wang et al. (2013), with some modifications. In brief, shoot tips (measuring approximately 2.0 mm in length) containing 4–5 leaf primordia (LP) were excised from three-week-old stock cultures without cold-hardening and were pre-cultured with MS supplemented with 0.3 M sucrose for one day. Pre-cultured shoot tips were loaded for 30 min at room temperature with a loading solution composed of MS medium containing 2 M glycerol and 0.4 M sucrose. After the loading process, shoot tips were dehydrated with plant vitrification solution 2 (PVS2) for 30 min at 0 °C. Dehydrated shoot tips were frozen and rewarmed accordingly, and post-cultured on SMM for shoot regrowth. The cultures were placed in dark conditions for the first three days and then transferred to light conditions, as used for *in vitro* stock culture. Subculture was done every three weeks.

2.3.3. Chemotherapy combined with thermotherapy

Chemotherapy combined with thermotherapy was conducted according to Gopal and Garg (2011). Briefly, shoot segments (0.5 cm in length) containing apical bud were excised from three-week-old stock cultures and placed on SMM supplemented with 20 mg/l ribavirin (Sigma-Aldrich Norway AS, Oslo, Norway), which was filter-sterilized and added to the medium after autoclaving. The cultures were kept in light conditions, as used for *in vitro* stock cultures. After three weeks of culture on ribavirin-containing medium, the cultures were moved into a growth chamber set at 37 ± 1 °C and 16 h photoperiod of 50 μmol $m^{-2}s^{-1}$ light intensity for two weeks of thermotherapy. Meristems (≤ 0.2 mm in length) containing 1–2 LP were excised and placed on sterile

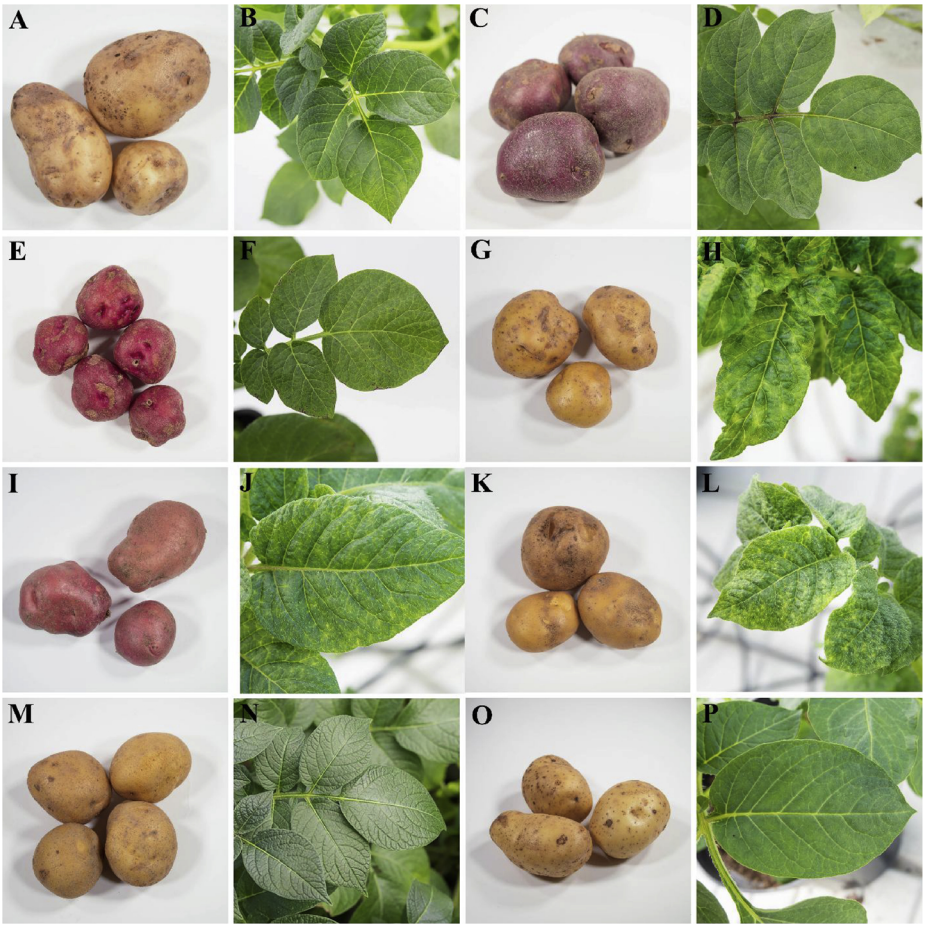

Fig. 2. Potato tubers and leaves infected with viruses. A and B: 'Truls'; C and D: 'Kerrs Pink blått skall'; E and F: 'Gammelraude'; G and H: 'Abundance'; I and J: 'Gjernespotet'; K and L: 'Hroar Dege'; M and N: 'Iverpotet/Smaragd'; O and P: 'Sverre'.

filter paper placed on MCM. The culture condition was the same used for *in vitro* stock cultures. Subculture was done every three weeks.

2.3.4. Shoot regeneration and establishment of plants in soil

Shoot regeneration was defined as shoots (≥ 1 cm) with at least two fully opened leaves developed after two months of post-culture from meristem culture and chemotherapy combined with thermotherapy, and one month from shoot-tip cryotherapy. Shoots formed well developed root systems after about six months of shoot regeneration. *In vitro* plantlets regenerated from the three *in vitro* therapies were tested using DAS-ELISA for their sanitary status, as described below. Samples showing negative response were considered virus-free and transferred to soil in an aphid-free greenhouse, which was set at 20 ± 2 °C under a 16-h photoperiod with a supplementary light intensity of 200 µmol s^{-1} m^{-2}. The plants were maintained in the aphid-free greenhouse for further confirmation of their sanitary status analyzed by DAS-ELISA, biological indicators and small RNA HTS.

2.4. Virus detection

Samples were tested for virus four times in the present study (Fig. 1), the first time by DAS-ELISA in the samples grown in a greenhouse, and showed symptoms. At the same time, total RNA was extracted from each single plant, pooled as one sample and tested with HTS. Samples were tested for virus a second time by DAS-ELISA after establishment of *in vitro* stock cultures for virus elimination. The third time, *in vitro* regenerants recovered from the three *in vitro* therapy treatments were tested for virus by DAS-ELISA after six months of regeneration. Samples showing a negative response to DAS-ELISA were considered virus-free and moved to soil in aphid-free greenhouse. Finally, sanitary status was confirmed by DAS-ELISA and biological indicators in the plants that were recovered from the three *in vitro* therapy treatments after two months of establishment in soil. The total RNA was then extracted from each single virus-free sample, pooled together, and detected by HTS. Virus elimination frequencies were registered based on negative reactions to DAS-ELISA and biological indicators.

2.4.1. DAS-ELISA

DAS-ELISA was performed according to Clark and Adams (1977). The viruses tested included potato virus A (PVA), potato virus Y (PVY), potato virus M (PVM), PVX, PVS and potato leafroll virus (PLRV). All antibodies were purchased from Bioreba (Reinach BL, Switzerland). Briefly, leaves (50 mg) were extracted with 5 ml phosphate-buffered saline (PBS, pH = 7.4). The first and second antibodies were diluted (1:1000) in coating and conjugate buffer. P-nitrophenyl phosphate was used as a substrate. Absorbance was recorded at 405 nm after incubation for 30–120 minutes. Samples doubling the reading data of the negative control were considered positive reactions to the tested viruses.

2.4.2. Biological indexing

All the virus-free plants tested by DAS-ELISA were inoculated on biological indicators. Six indicators – *Solanum demissum* A (SdA), *Solanum demissum* Y (SdY), *Nicotiana debneyii* Domin, *Chenopodium quinoa* Willd and *Capsicum annum* L. – propagated by true seeds were used according to the Norwegian regulations for production of seed potatoes (Forskrift om settepoteter, http://lovdata.no) and EPPO (1999). All indicator plants were mechanically inoculated at the 4–6 leaf stage with sap from about 100 mg of fresh leaves grinded in 400 µl 0.03 M PBS (pH7.0) buffer (Bos, 1999). All plants were grown in an aphid-free greenhouse at 20 ± 2 °C with a 16-h photoperiod and a

Table 1
Shoot regeneration and virus elimination by meristem culture of potato.

Cultivar	Shoot regeneration (%)	Frequency of virus-free plants (%)				
		PVY	PVM	PVS	PVX	All viruses*
Truls	70	100	NI**	36	7.1	7.1
Kerrs Pink blått skall	60	NI	NI	16.7	NI	16.7
Gammelraude	60	NI	0	16.7	NI	0
Abundance	50	NI	NI	NI	0	0
Gjernespotet	40	NI	NI	37.5	25	0
Hroar Dege	75	NI	NI	0	NI	0
Iverpotet/ Smaragd	80	NI	NI	12.5	NI	12.5
Sverre	75	66.7	NI	6.7	NI	6.7
Overall mean (%)	63.8	82.8	0	16.3	9	

* Frequency of regenerated plants free of all viruses.
** NI = no infection.

supplementary light intensity of 200 μmol s^{-1} m^{-2} before and after inoculation. The symptom observations started four–five days after inoculation and were carried out for six–eight weeks.

2.4.3. HTS

Total RNA was extracted with a Norgen Plant/Fungi RNA kit (Norgen Biotek, Thorold, ON, Canada) from fresh young leaf tissue (50 mg) according to the manufacturer's instructions. On-column DNase treatment was applied during RNA extraction. RNA quantity and quality were assessed by a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA samples with RIN (RNA integrity number) scores greater than 7.0 were used for HTS. A small RNA library was prepared and sequenced with Illumina HiSeq 2500 (Fasteris SA, Geneva, Switzerland). Sequencing results were analyzed with VirusDetect (Zheng et al., 2017) with 18–33bp reads selected.

2.5. Virus effect on the vegetative growth of potato in vitro cultures and microtuber production

Potato 'Truls' shoots recovered from *in vitro* therapies (as described above) and the virus infection status analyzed by DAS-ELISA were used in this experiment. Shoot segments (about 0.5 cm in length) were excised from shoots of the virus-free PVX, PVS, PVY, PVX + PVS, and PVX + PVY + PYS-infected potato 'Truls' after six months of regeneration, cultured on SMM under the same culture conditions as those used for *in vitro* stock cultures. Bud break was recorded after three days, node number and plant heights were recorded after three weeks of culture.

In vitro microtuber production was conducted according to Li et al. (2013). Nodal segments (1.0 cm in length) containing a single leaf and axillary bud were taken from shoots of the virus-free PVX, PVS, PVY, PVX + PVS, PVX + PVY + PYS-infected potato 'Truls'. The segments were cultured in a microtuber induction medium containing SMM supplemented with 60 g/l sucrose and 4 mg/l kinetin in dark conditions. The number of microtubers was recorded after eight weeks of culture.

2.6. Experimental design and data analysis

For *in vitro* therapies, 10 samples were included in each treatment of two replicates from each cultivar. The whole experiment was repeated twice. The number of samples used for virus detection varied depending on available samples obtained in the experiments. The virus elimination frequency was defined as the number of regenerated plants showing negative responses to both DAS-ELISA and biological indicators/total number of samples tested × 100%.

For the virus effect on vegetative growth of potato *in vitro* cultures and microtuber production, 10 shoots were included in each glass and three glasses of each sample were used. The whole experiment was repeated twice. The data were analyzed by Student's *t*-test ($P \leq 0.05$).

3. Results

3.1. Virus infection and symptomatology

The virus infection status of the eight potato cultivars was screened using DAS-ELISA. The symptoms observed in each cultivar are presented in Fig. 2. Potato 'Abundance' was single-infected with PVX, and 'Kerrs Pink blått skall', 'Hroar Dege' and 'Iverpotet/Smaragd' were single-infected with PVS. Three potato cultivars were co-infected: 'Gammelraude' with PVM and PVS, and 'Gjernespotet' and 'Sverre' with PVY and PVS. Potato 'Truls' was mix-infected with PVY, PVS and PVX. Regarding symptoms, leaf mottles were found on fully opened leaves of 'Kerrs Pink blått skall' (Fig. 2D), while obvious mosaic and vein yellowing symptoms were found on fully opened leaves of 'Hoar Dege' (Fig. 2L). Leaves on 'Abundance', (Fig. 2H) showed obvious mosaic and vein yellowing symptoms and single infection of PVX. In co-infected cultivars, mottles and necrotic spots were observed on the fully opened leaves of 'Gammelraude' (Fig. 2F); potato 'Sverre' fully opened leaves showed vein yellowing and mild mottle symptoms (Fig. 2P); leaves on 'Gjernespotet' (Fig. 2J) showed obvious mosaic and vein yellowing symptoms. Leaf mottles were observed on fully opened leaves of 'Truls' (Fig. 2B) and mix-infection of PVY, PVS and PVX. No obvious symptoms were observed on any leaves on 'Iverpotet/Smaragd' (Fig. 2N).

3.2. In vitro therapies

3.2.1. Meristem culture

Meristems of the eight potato cultivars tested were able to regenerate shoots, and their regeneration levels ranged from 80% in potato 'Iverpotet/Smaragd' to 40% in potato 'Gjernespotet' (Table 1). One potato 'Truls', two 'Kerrs Pink' and one 'Sverre' were found to be free of all the viruses (Table 1). The overall mean virus elimination rates for single viruses were 82.8% for PVY, 16.3% for PVS, 9% for PVX and 0% for PVM (Table 1).

3.2.2. Shoot-tip cryotherapy

After shoot-tip cryotherapy of the eight potato cultivars tested, regeneration rates of between 30% and 70% were obtained, the lowest of which was for potato 'Kerrs Pink blått skall' and the highest for 'Hroar Dege' (Table 2). The overall mean elimination rates for single viruses were 100% for PVY, 3.2% for PVX, 2.8% for PVS and 0% for PVM (Table 2). Altogether, two 'Iverpotet/Smaragd' plantlets were found to be virus-free (Table 2).

3.2.3. Chemotherapy combined with thermotherapy

After chemotherapy combined with thermotherapy was applied, the regeneration rates for all cultivars varied from 15% to 72.5%, the lowest of which was for potato 'Kerrs Pink blått skall' and the highest for 'Abundance' (Table 3). After confirmation by both DAS-ELISA and indicator plants, 50% of 'Truls', 100% of 'Kerrs Pink blått skall', 85.7% of 'Gammelraude', 96.6% of 'Abundance', 36.4% of 'Gjernespotet', 83.3% of 'Hroar Dege', 94.7% of 'Iverpotet/Smaragd' and 100% of 'Sverre' plants were virus-free (Table 3). The overall mean elimination rates for single viruses were 85.7% for PVY, 92.9% for PVM, 84.5% for PVS and 74.2% for PVX (Table 3).

3.3. Virus test by HTS

HTS of small RNA were applied to two samples: a sample pool of

Table 2
Shoot regeneration and virus elimination by shoot tip cryotherapy of potato.

Cultivar	Shoot regeneration (%)	Frequency of virus-free plants (%)				
		PVY	PVM	PVS	PVX	All viruses*
Truls	50	100	NI**	0	0	0
Kerrs Pink blått skall	30	NI	NI	0	NI	0
Gammelraude	45	NI	0	0	NI	0
Abundance	40	NI	NI	NI	0	0
Gjernespotet	65	NI	NI	0	7.7	0
Hroar Dege	70	NI	NI	0	NI	0
Iverpotet/ Smaragd	60	NI	NI	16.7	NI	16.7
Sverre	40	100	NI	0	NI	0
Overall mean (%)	50	100	0	2.8	3.2	

* Frequency of regenerated plants free of all viruses.
** NI = no infection.

Table 3
Shoot regeneration and virus elimination by combining chemotherapy with thermotherapy of potato.

Cultivar	Shoot regeneration (%)	Frequency of virus-free plants (%)				
		PVY	PVM	PVS	PVX	All viruses*
Truls	65	76.9	NI**	61.5	61.5	50
Kerrs Pink blått skall	15	NI	NI	100	NI	100
Gammelraude	35	NI	92.9	92.9	NI	85.7
Abundance	72.5	NI	NI	NI	96.6	96.6
Gjernespotet	27.5	NI	NI	81.8	45.5	36.4
Hroar Dege	45	NI	NI	83.3	NI	83.3
Iverpotet/ Smaragd	47.5	NI	NI	94.7	NI	94.7
Sverre	32.5	100	NI	100	NI	100
Overall mean (%)	42.5	85.7	92.9	84.5	74.2	

* Frequency of regenerated plants free of all viruses.
** NI = no infection.

potato plants grown in greenhouse and showing symptoms (virus-infected sample), and a sample pool of potato plants recovered from the three *in vitro* therapy treatments after two months of establishment in soil in an aphid-free greenhouse and showing negative response to DAS-ELISA and biological indicators (virus-free sample). 474 Mb and 2, 461 Mb reads were generated for the virus-infected sample and the virus-free sample, with 94.59% and 97.01% passed filter respectively. The size distribution of reads after adapter trimming of HTS data are shown in Table 4, 18–26bp were most abundant, with 57.19% in the virus-infected sample and 74.18% in the virus-free sample (Table 4). The size window of 18 to 26 bases is of interest because it corresponds to small RNA. Two peaks were observed, at 21 bp and 24 bp respectively (data not shown).

VirusDetect was used to analyze the sequence of the virus-infected sample, and adapter-trimmed 18–33 bp reads (5,941,062) were selected, 52 virus accessions were identified by BLASTN (nucleotide similarity) and one virus accession was identified by BLASTX (protein similarity). Altogether, PVM, PVS, PVX, PVY (Fig. 3, Table 4) were found.

VirusDetect was used to analyze the sequence of the virus-free sample, and adapter-trimmed 18–33 bp reads (44,102,063) were selected, no virus were found by blasting contigs with BLASTN and BLASTX, which is consistent with the DAS-ELISA and biological indicator results.

3.4. Virus effect on vegetative growth of potato in vitro cultures and microtuber production

Different virus infections had significant effects on the vegetative growth of the *in vitro*-cultured potato 'Truls'. After three days of culture, the bud-break percentages of healthy segments, PVX, PVS and PVY single-infected, PVX and PVS co-infected segments were 90%, 83%, 80%, 73% and 77% respectively (Fig. 4A). But PVX, PVS and PVY co-infected segments showed 10% bud-break, which differed significantly from the others (Fig. 4A).

After three weeks of culture, the longest plant height (10.1 cm) was found in healthy segments, followed by PVX single-infected (9.3 cm), PVS single-infected (8.7 cm), PVX and PVS co-infected (8.6 cm), and PVY single-infected segments (8.5 cm) (Fig. 4B). PVX, PVS and PVY triple-infected segments showed significantly lower plant height (5.2 cm) (Fig. 3B). PVX, PVS and PVY triple-infected segments resulted in the production of most nodes (8), and PVS single-infected segments resulted in the least nodes (4.3), thereby differing significantly from the other four segment types. Healthy segments, PVX, PVY, PVX and PVS co-infected, resulted in the production of 5.5, 5.2, 5.7 and 5.6 nodes, respectively (Fig. 4C).

Viral diseases exerted negligible influence on microtuber production of *in vitro* cultures of potato 'Truls' segments except in the case of triple infection of PVX, PVS and PVY (Figs. 4D and 5). Triple infection potato 'Truls' produced smaller microtubers (Fig. 5E) and significantly fewer microtubers (3) per glass (Fig. 4D). All the other segment types produced between 9.3 and 11.7 microtubers per glass, showing no significant differences (Fig. 4D). Compared with others (Fig. 5A–E), the size of the microtubers produced by healthy segments (Fig. 5F) was equal or larger.

4. Discussion

The present study tested three *in vitro* therapies for eliminating potato viruses from eight potato-valuable germplasm in Norway. With these three methods, 30–80% shoot regeneration and up to 100% virus-free frequency were obtained. Chemotherapy combined with thermotherapy proved the most effective method of virus elimination from all the eight potato cultivars tested, and can be considered effective in the production of virus-free seed potatoes in Norway. Whatever, successful establishment of three therapeutic methods in the present study provided options to use in laboratories working on virus elimination, depending on availability of any method in a given laboratory. In

Table 4
The size distribution of inserts after adapter trimming of Illumina high-throughput sequencing data and analyzed by VirusDetect.

Insert range (bp)		0	1-17	18-26	27-50	VirusDetect
Virus-infected sample[1]	No. of reads	351'816	3'089'710	5'417'442	614'262	PVM, PVS, PVX, PVY,
	% reads	3.71	32.62	57.19	6.48	
Virus-free sample[2]	No. of reads	2'522'038	2'092'318	36'519'995	8'094'884	No virus
	% reads	5.12	4.25	74.18	16.44	

[1] Sample pool of potato plants grown in greenhouse and showing symptoms.
[2] Sample pool of potato plants recovered from the three *in vitro* therapies after two months of establishment in soil in aphid-free greenhouse and showing negative response to DAS-ELISA and biological indicators.

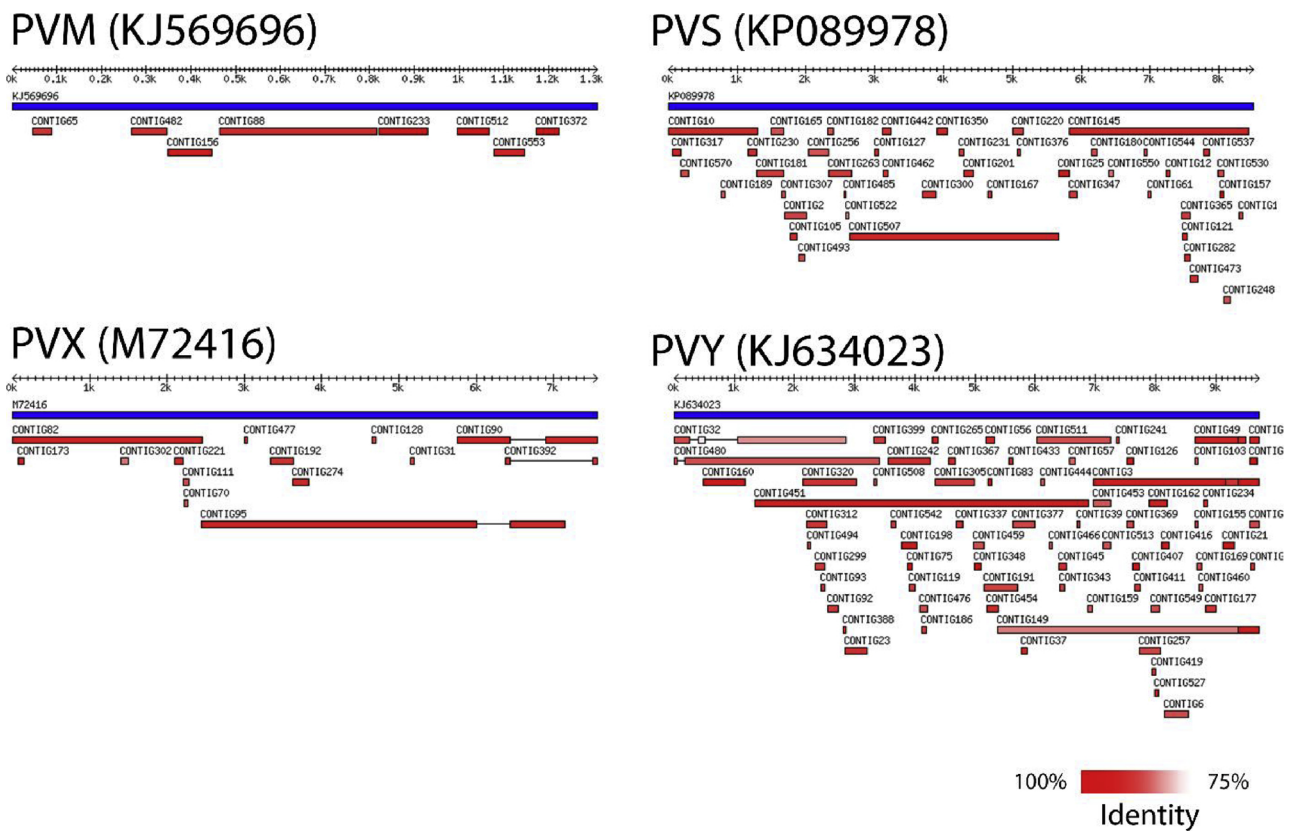

Fig. 3. Viruses identified in potato plants grown in greenhouse and showing symptoms (virus-infected sample) by small RNA high-throughput sequencing and data analyzed by VirusDetect. Alignments of identified virus contigs to the reference virus genomes. Blue tracks represent reference virus genomes [with National Center for Biotechnology Information (NCBI) genebank number], and red tracks represent assembled virus contigs. PVS, potato virus S; PVM, potato virus M; PVY, potato virus Y; PVX, potato virus.

addition, HTS was used for the first time to confirm the virus status in the plants resulting from *in vitro* therapy, thus ensuring accurate verification of the virus status of valuable potato germplasm.

Cryopreservation of potato shoot tips has been extensively studied, and a routine procedure for the cryopreservation of more than 60 genotypes has been developed in Norway. In practice, cryopreservation is a good method to combine potato conservation and virus elimination (Wang et al., 2006). In this study, droplet-vitrification cryopreservation protocol had a good regeneration rate (up to 70%) and was very efficient in eradicating PVY (100%). However, PVM, PVS and PVX were almost impossible to be eradicated (up to 3% elimination rate). Li et al. (2018b) reported that droplet-vitrification cryotherapy of both 0.5 mm and 1.5 mm shoot tips was not effective in eradicating PVS from potato 'Zihuabai'. Kushnarenko et al. (2017) also reported that cryotherapy alone resulted in 38.6% virus-free potato shoots in accessions infected only with PVM, but cryotherapy was not effective in accessions that were mix-infected with PVM, PVS or PVY.

Ribavirin (1-β-D-ribofurasonyl-1,2,4 triazone-3-carboxamide), synthesized in 1972, has been commonly used to eradicate viruses from potato (Klein and Livingston, 1983; Yang et al., 2014). Klein and Livingston (1983) reported that PVX and PVY were eliminated by ribavirin chemotherapy combined with meristem culture, while the time required for plantlet development was delayed by up to three months. Gopal and Garg (2011) reported that chemotherapy with 20 mg/l ribavirin and thermotherapy followed by meristem culture was efficient in eradicating PVX and PVS. In our study, stock plants were cultured on medium containing 20 mg/l ribavirin for three weeks before thermotherapy for up to two weeks. The virus eradication rates for single viruses were 85.7% for PVY, 92.9% for PVM, 84.5% for PVS and 74.2% for PVX, respectively. This is a method with low concentration of ribavirin, high efficiency and less time consuming for potato virus eradication, and suitable for variable potato cultivars.

Potato species are hosts of at least 39 viruses which naturally infect cultivated potatoes (Lacomme and Jacquot, 2017). PVX, PVY, PVS and PVM commonly occur in potato crops in Norway. Single or mixed infection of viruses can cause significant yield and quality losses (Wang et al., 2011; Yang et al., 2014). In our study, we found that the potato 'Abundance', 'Hroar Dege' and 'Gjernespotet' were highly sensitive to single infection of either PVX or PVS, and to co-infection of PVX and PVS, based on the observation of obvious and serve mosaic symptoms appeared on leaves. On the other hand, potato 'Iverpotet/Smaragd' showed almost no symptoms when infected with PVS. Potato 'Truls', which was infected with three different viruses (PVY, PVX and PVS) showed only a few leaf mottles on the fully open leaves. In addition, most of the potato cultivars (except potato 'Truls') used in this study have been grown in the same field, with tuber multiplication from year to year, without infection of PVY. PVY is one of the viruses most easily transmitted *via* infected tubers, insect vectors or other means from one generation to the next (Scholthof et al., 2011; Wang et al., 2011). These symptoms/facts may indicate that these potato cultivars probably contain virus tolerance that can be valuable for breeding new cultivars.

Plant diseases caused by viruses can be effectively controlled when planting virus-free crops. The diagnosis is the basis for managing plant diseases and for predicting crop losses due to infection of plant pathogens (van der Want and Dijkstra, 2006). As a vegetative propagated crop, potato is particularly susceptible to virus infection carried from one generation to the other through seed potato. This is why accurate diagnosis is important for verifying the virus status of valuable potato germplasm. The tissue-culture germplasm and seed potatoes used for production are routinely tested for virus infection with a 'zero' infection tolerance for a certain number of viruses (Wilson, 2014). But more mechanically transmitted viruses can be presented in addition to all the

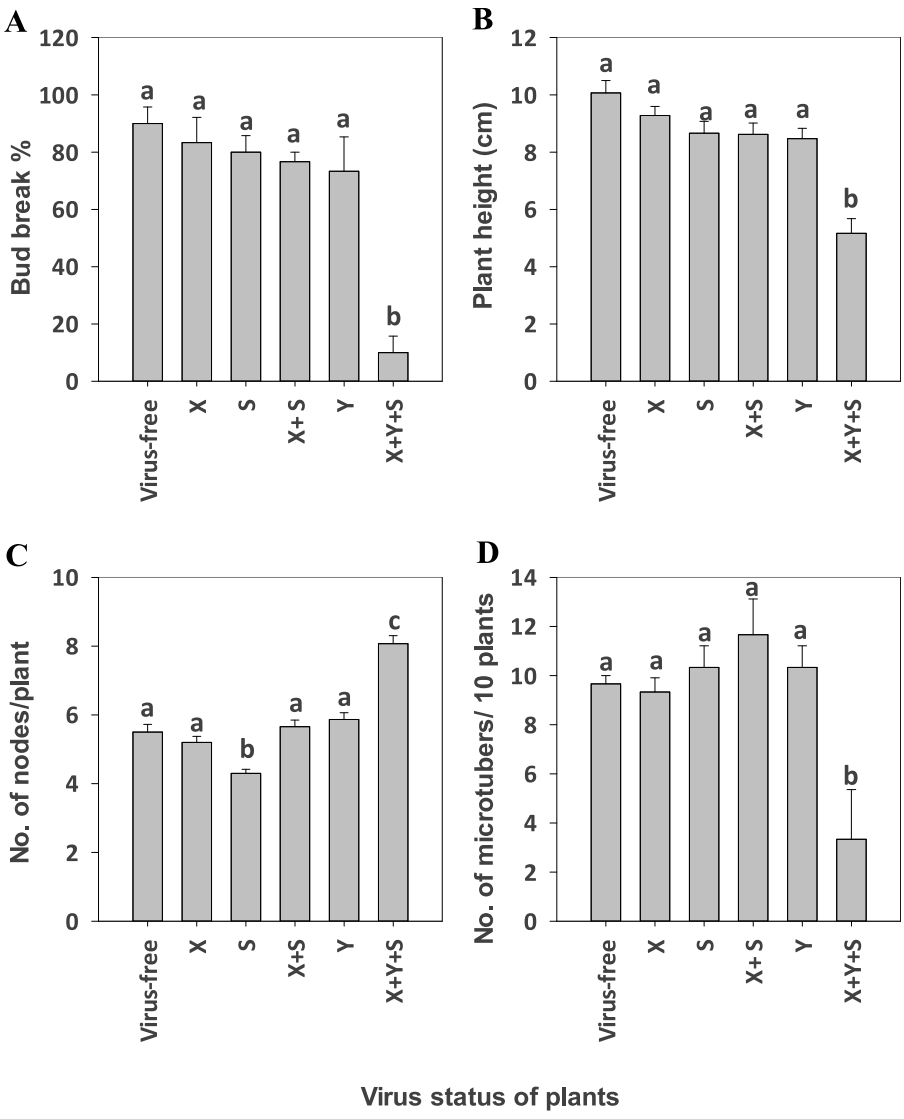

Fig. 4. Effect of virus infection on vegetative growth and microtuber production of *in vitro* segments of potato 'Truls'. Bud-break was recorded after three days of culture (A), while plant height (B) and number of nodes were recorded after three weeks of culture (C). Number of microtubers was registered after eight weeks of culture (D). 'X': single infection of PVX; 'S': single infection of PVS; 'X + S': co-infection of PVX and PVS; 'Y': single infection of PVY; 'X + Y + S': triple infection of PVX, PVY and PVS. The data were presented as means ± SE and with different letters in the same parameter indicating significant differences at $P \leq 0.05$ by Student's *t*-test.

tested ones. There is increased interest in applying HTS approaches to virus detection, as well as to official plant inspection. HTS is gaining popularity for screening a wide range of viruses simultaneously, and can detect all viruses without the need for specific virus antiserum or virus primers or probes (Kreuze et al., 2009; Olmos et al., 2018). HTS analyses have the potential to eventually replace lengthy and tedious resource-intensive biological indexing and speed up the flow pace of novel varieties for growers (Olmos et al., 2018). Al Rwahnih et al. (2015) reported that HTS has been found to be superior to standard bioassays for the optimal detection of viral pathogens in grapevine. In this respect, testing candidate materials by HTS could be an efficient diagnostic strategy in the preservation of valuable potato germplasm or even seed potato certification, to detect unknown or unexpected viruses, compared with ELISA, PCR or other molecular diagnostic methods (Olmos et al., 2018; Santala and Valkonen, 2018).

The results of virus infection on vegetative growth and the

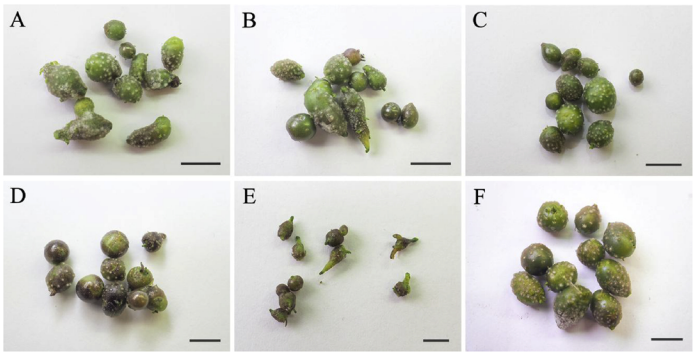

Fig. 5. Microtubers of potato 'Truls' infected with PVX (A), PVS (B), co-infection with PVX and PVS (C), single infection of PVY (D), co-infection with PVX, PVS and PVY (E) and virus-free (F). Scale bars are 1 cm.

microtuber production of *in vitro* cultures of potato 'Truls' clearly demonstrated that the inhibitory effect of PVY when co-infected with PVX and PVS was much stronger than any single infection with either PVS, PVX or PVY or co-infection of PVX and PVS. Nyalugwe et al. (2012) also reported that single infection with PVX or PVS and co-infection of PVX and PVS showed no significant differences in tuber yield. Nonetheless, previous studies also reported that co-infection/triple infection of different viruses could lead to more serious effects on vegetative growth in *in vitro* culture systems. Li et al. (2013; 2018a,b) found that PLRV and PVY co-infection had a much stronger effect than any single infection with either PLRV or PVY on vegetative growth. Tsao et al. (2000) found that the inhibitory effect of co-infection with *Raspberry bushy dwarf virus* (RBDV) and *Tobacco streak virus* (TSV), or RBDV and Tomato ringspot virus (TomRSV), or mix-infected with three viruses (RBDV + TomRSV + TSV) on raspberry (*Rubus idaeus*) was much stronger than in those single-infected with either RBDV or TomRSV or TSV.

In conclusion, three *in vitro* therapies – meristem culture, shoot-tip cryotherapy and chemotherapy combined with thermotherapy – were successfully established for virus elimination in the eight potato old cultivars representing the valuable potato germplasm in Norway. Chemotherapy combined with thermotherapy was the most effective of the three *in vitro* therapies used in this study. The results reported here provide a technical platform for establishing virus-free potato germplasm. Small RNA HTS has been reported for the first time to evaluate virus status after virus eradication and production of virus-free nuclear stocks of potatoes.

Author contributions

Z. Zhang: performance of experiments, data collection and analysis, and preparation of manuscript; Q.-C. Wang: valuable discussion and revision of manuscript; C. Spetz: valuable discussion; D.-R. Blystad: project management, design of experiments and revision of manuscript. All the authors have read and approved the final manuscript.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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