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Biological efficacy of chitosan nanoparticles and black poplar buds ethanolic extract against potato dry rot

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Abstract. The purpose of the recent study was to evaluate the effectiveness of chitosan nanoparticles (CSNP) and black poplar buds extract (BpE) to protect potato tubers from post-harvest *Fusarium* dry rot. A high virulent *Fusarium* isolate was recovered from natural infected tubers in Astrakhan region and subjected to molecular identification using ITS rDNA gene and identified as *Fusarium sambucinum*. CSNP were successfully prepared by ionic gelation method with size ranged from 79.4 to 186.4 nm as observed by scanning electron microscopy (SEM), and a positive zeta potential of 53.1 mv. CSNP at a concentration of 2 g/l, and BpE at a concentration of 40 g/l completely inhibited *Fusarium* fungal growth on PDA medium *in vitro*. CSNP at a rate of 20 g/t and BpE at a rate of 400 g/t significantly reduced the incidence of dry rot under natural infection during 8 months of storage by 54.7 and 58.5% and reduced the disease index by 65.7 and 72.3%, respectively, and there was not a significant difference between the two treatments. These results demonstrate the potential of CSNP and BpE for protection of potato tubers against the fungal postharvest decay.

Keywords: Nanotechnology, *Fusarium*, plant diseases, antifungal, plant extracts

Author contributions. Zeitar E.M., Mohammed S.R. — formation of the research idea, selection and carry out of the research methods, results analysis, preparation of the manuscript; Sukhenko L.T. — visualization, supervision, revision.

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Биологическая эффективность наночастиц хитозана и экстракта почек тополя черного в отношении сухой гнили картофеля

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Аннотация. Цель исследования — оценка эффективности наночастиц хитозана (CSNP) и экстракта почек тополя черного (ВрЕ) для защиты клубней картофеля от послеуборочной фузариозной сухой гнили. Высоковирулентный изолят *Fusarium* выделен из зараженных естественным путем клубней в Астраханской области, подвергнут молекулярной идентификации с использованием гена ITS-rDNA и идентифицирован как *Fusarium sambucinum*. CSNP успешно получены методом ионного гелеобразования с размером в диапазоне от 79,4 до 186,4 нм, наблюдаемым с помощью сканирующей электронной микроскопии (СЭМ), и положительным дзета-потенциалом 53,1 мВ. CSNP при концентрации 2 г/л и ВрЕ при концентрации 40 г/л полностью подавляли рост грибов рода *Fusarium* на среде КДА *in vitro*. CSNP в дозе 20 г/т и ВрЕ в дозе 400 г/т значительно снизили частоту сухой гнили при естественном заражении в течение 8 месяцев хранения на 54,7 и 58,5 %, а также снизили индекс заболеваемости на 65,7 и 72,3 % соответственно, и между этими двумя обработками не было существенной разницы. Эти результаты демонстрируют потенциал наночастиц хитозана и экстракта почек тополя черного для защиты клубней картофеля от грибковой послеуборочной гнили.

Ключевые слова: нанотехнологии, фузариоз, болезни растений, противогрибковое средство, растительные экстракты

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Introduction

Post-harvest potato dry rot caused by *Fusarium* spp. has the potential to be a devastating disease in various parts of the world [1]. Surface sterile in 10% sodium hypochlorite for 10 s, rinsed twice in sterile distilled water, and blotted with sterile filter paper. The tissue pieces were plated on half-strength potato dextrose agar (PDA). It could affect tubers during storage and seed pieces in the field [2]. Losses by this disease are between 6 to 25%, and could reach up to 60% [3]. The chemical fungicides application are the main method to control dry rot [4].

Considering to escalate pathogen resistance to fungicides and their toxic effects on the environment and human, it has become imperative to identify novel natural substances by utilizing innovative technologies [5]. Avoiding the chemicals use could be achieved by introducing plant extracts in plant protection field [6].

The black poplar (*Populus nigra* L.), a member of the Salicaceae family, is considered an abundant tree in deciduous forests. Many of its parts and residues can be bio-resources for active ingredients in medical applications [7]. *P. nigra* buds have been characterized as a vital source of phenolics and terpenoids, which are responsible for the biological effectiveness [8]. *P. nigra* leaf extract improved the growth parameters and fruit quality of *Capsicum annuum* infected with pepper mild mottle virus [9].

Chitosan, alone or in combination with other chemical agents showed a significant effect on plant development, tuber yield, and disease incidence under field conditions against potato fungal pathogens [10, 11]. It possesses innate antimicrobial potential and induces the release of hydrolytic enzymes in plant parts, which actively contribute to plant defense against a range of pathogens [12].

Chitosan nanoparticles (CNP) have emerged as a significant innovation in plant protection, offering a sustainable alternative to traditional chemical pesticides. Their unique properties, including biodegradability and non-toxicity, enable them to enhance plant health and productivity while minimizing environmental impact.

The purpose of the research was to evaluate the biological effectiveness of chitosan nanoparticles and black poplar buds extract to protect potato tubers from *Fusarium* dry rot under long storage conditions.

Materials and Methods

Chitosan with a molecular weight of 150 kDa, and deacetylation degree 85% was purchased from Chitosan-Technology company. Sodium tripolyphosphate (STPP), and glacial acetic acid were purchased from Len-Reactiv (Saint Petersburg, Russian Federation). The components of black poplar were extracted by percolation in 70% ethanol [13] (Fig. 1). Potato tubers of variety Rivera obtained from local commercial farm.



Fig. 1. *Populus nigra*: *a* – buds; *b* – ethanolic extract
Source: compiled by E.M. Zeitar, L.T. Sukhenko, S.R. Mohammed.

Isolation and morphological identification of the pathogen. The fungal pathogen was isolated from natural infected tubers (Fig. 2) on PDA medium [5, 14]. Colony features of color, shape, edge, elevation and radial growth were visually examined from the top and back of pure cultures. For further verification, the vegetative and reproductive structures of the isolates (macroconidia, microconidia and chlamydospores) were examined under a compound microscope with 40x magnification by using the morphological key [15].



Fig. 2. Typical symptoms of *Fusarium* dry rot: *a* – in potato tubers; *b* – longitudinal section
Source: compiled by E.M. Zeitar, L.T. Sukhenko, S.R. Mohammed.

Molecular identification of fungal Isolate. *DNA extraction:* Genomic DNA extraction was performed from 7-day fresh cultures using a genomic DNA mini kit (Geneaid) according to the manufacturer’s protocol.

PCR amplification: The ITS region of the rDNA repeats from the 3’ and 5’ ends of the 28S gene was amplified using the two primers, ITS-1 and ITS-4 which were synthesized by MWG-Biotech, Germany based on conserved regions of the eukaryotic rRNA gene [16]. Details of the primers are clarified in Table 1.

Table 1

Primers sequences used in amplification of ITS rDNA region of the fungal isolate

Primer name	Sequence 5’→3’	Reference
ITS1 (Forward)	TCCGTAGGTGAACCTGCGG	[17]
ITS4 (Reverse)	TCCTCCGCTTATTGATATGC	

Source: [17].

DNA sequencing of fungal ITS rDNA gene: For each fungal isolate, after purification of ITS fragments, excised the target band and purified by DNA gel Extraction Kit (Fermentas, Germany), and the purified PCR products were sequenced by BigDye Terminator Cycle Sequencing kit (Perkin Elmer) on an ABI Prism 310 Genetic Analyzer (Applied Biosystems) by Macrogen (macrogen com, Korea) and deposited the generated sequences in the GenBank (EMBL database).

Sequence analysis and phylogeny: The consensus sequences of fungal isolate obtained from both ITS1 and ITS4 primers were first edited and subjected to BLAST search to assign putative identity with similar sequences using database of NCBI <http://blast.ncbi.nlm.nih.gov/Blast.cgi>. Each fungal isolate was then designed to its operational taxonomic unit (OTU) based on measures of sequence similarities, and inferences of phylogenetic trees. Sequences were then aligned with other similar sequences downloaded from GenBank using ClustalX [18], BioEdit [19] and Molecular Evolutionary Genetics Analysis (MEGA) software ver. 6.0 [20]. Alignments were manually edited where necessary. All nucleotide sequences of the pathogenic fungi have been submitted to GenBank for accession numbers.

Sequences obtained were split into different datasets to assess phylogenetic relationships at the familial and species level. Phylogenetic trees were constructed using neighbor Joining (NJ) method and molecular evolutionary analyses were conducted using MEGA software, ver. 11.0 [20].

Preparation of Chitosan nanoparticles. CSN were prepared by the ionic gelation method as described by [21] with chitosan solution (1%).

Morphology, size and Zeta potential of nanoparticles. The morphology of nanoparticles was studied using scanning electron microscopy (SEM) (TESCAN VEGA,

Czech Republic). The dried nanoparticles (1 mg) were dispersed in deionized water (20 ml) and treated with ultrasound for 10 minutes. One drop of dispersion containing chitosan nanoparticles was applied to a silicon wafer and dried at room temperature. Then the dried nanoparticles were coated with carbon under a high vacuum. The zeta-potential of freshly prepared nanoparticles in suspension were measured using a Malvern nano-series Zeta-sizer (UK) with a helium-neon laser operating at a scattering angle of 90° and a wavelength of 633 nm at 25 °C. Three replicate samples were analyzed, and the mean value was reported.

Antifungal activity on mycelial growth. Antifungal assay was performed with the pour-plate method as described by [22], and the mycelial growth was measured within 7–10 days after inoculation. Mycelial growth inhibition was calculated as follows:

$$\text{Mycelial growth inhibition \%} = \frac{C - T}{C} \times 100,$$

where C and T are the mycelial radial growth, mm, of fungus in the control and treatments, respectively.

Biological efficacy on potato tubers under natural infection. The effectiveness of chitosan nanoparticles and black poplar buds extract against *Fusarium* dry rot was evaluated during storage period on the Riviera variety grown with natural infection in the field of “KFH Jafarov Nazhmudin Vagidovich” (Limansky district, Astrakhan region, Russian Federation, 2020–2022) up to 8 months at 4 °C, the selection of tubers for the experiment was carried out 7 days after harvesting potatoes. Tubers were treated using a UNITRAUM sprayer before laying potato in storage, followed by natural drying, at the following rate of application: CSNP 20 g/t; BpE 400 g/t; Chi 100 g/t, with a working fluid consumption of 10 l/t. Each treatment had 10 kg tubers (placed in a grid mesh) and four replications [23]. The disease index X according to [24] calculated by the formula

$$X = \frac{dh}{DH} \times 100,$$



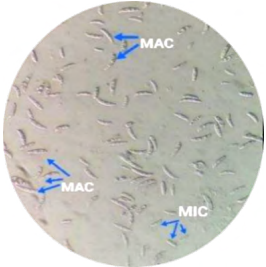

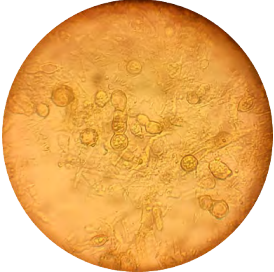


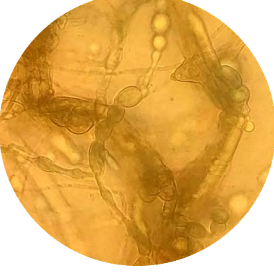
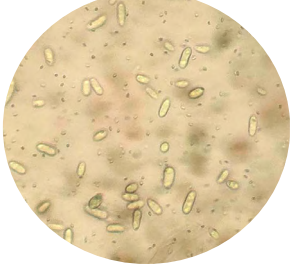

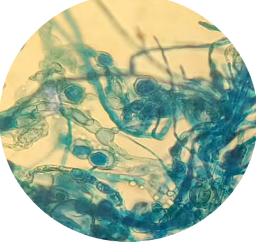
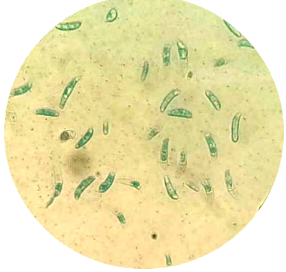
where d and h are the lesion (rot) width, and depth, mm; D and H are the tuber width, and depth, mm.

Results and discussion

Morphological and molecular identification of *Fusarium* spp. For morphological identification of the pathogen, fungal isolates forming salmon-pink, white-pink, and white colonies on potato-dextrose agar after 7–14 days of incubation and producing curved macroconidia were identified as *Fusarium* sp. The conidia were uniform in type and size. Macroconidia were abundant, with 3–5 septa, with a pointed apical cell. Microconidia were rare, elliptical, with partitions 0-1. Isolates produced chlamydospores singly or in pairs (Table 2), and these results are similar to the results obtained by [25] and [26].

Table 2

Morphological features of different *Fusarium* species recovered from potato tubers

Isolates key	The appearance of the colonies	Chlamydospores	Macroconidia (MAC), Microconidia (MIC)
FA3			
FA26			
FR23			
FS12			

Note. (FA3, FA 26) *Fusarium sambucinum*, (FR 23, FS12) *Fusarium solani*.
Source: compiled by E.M. Zeitar, L.T. Sukhenko, S.R. Mohammed.

According to the pathogenicity test, the most aggressive isolate was subjected to molecular identification using sequencing of ITS rDNA gene (Fig. 3). The obtained ITS consensus sequence of fungal isolate, was compared with the available sequences

GenBank (NCBI, <https://www.ncbi.nlm.nih.gov/>). The isolate exhibited relatedness to the genus *Fusarium*. The phylogenetic analysis revealed that strain SSD-MREZ had high similarity with *Fusarium sambucinum* strain Fsa0553-P isolated from potato tuber in Poland and *Fusarium sambucinum* isolate TR1 isolated from potato in India. The achieved sequence was submitted to GenBank with accessed accession number OR144017. The phylogenetic tree was constructed by MEGA software with the closely related *Fusarium* species as presented in Fig. 3.

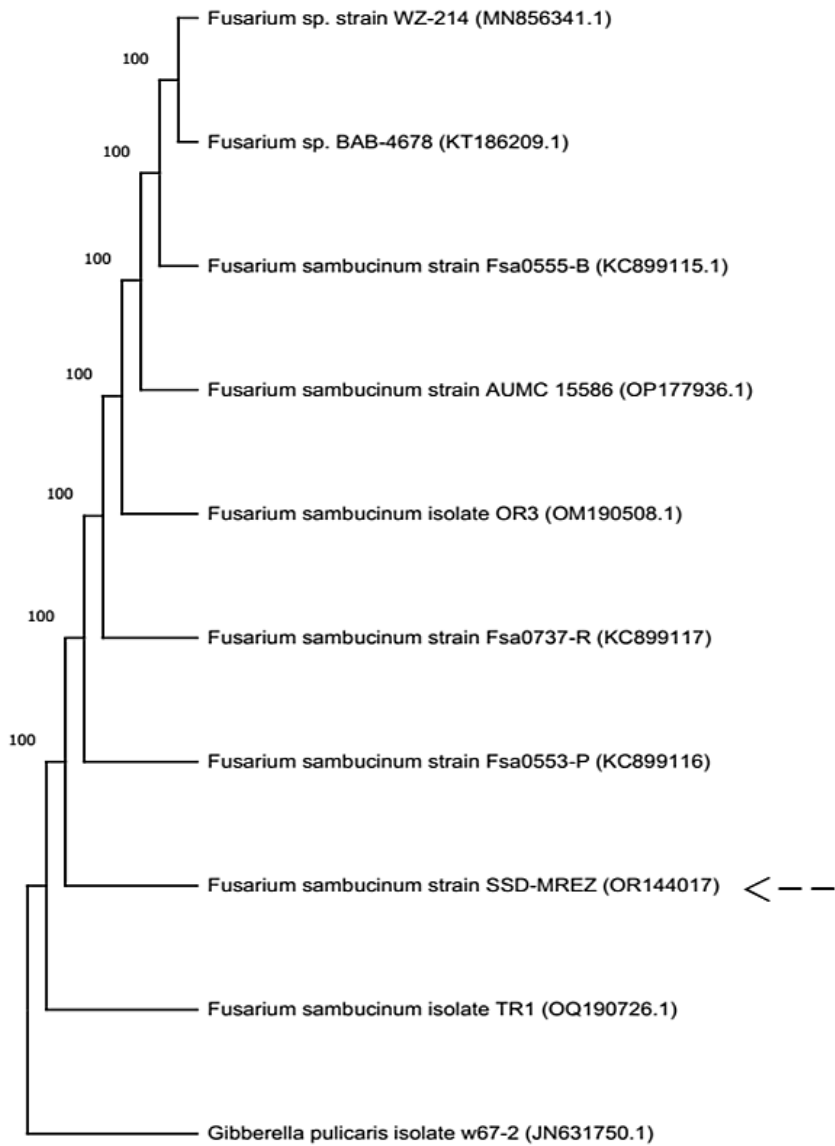


Fig. 3. Phylogenetic tree of *Fusarium* isolate based on ITS sequence data. The number above branches indicate bootstrap values

Source: compiled by E.M. Zeitar, L.T. Sukhenko, S.R. Mohammed.

Morphology, size and ZP of nanoparticles. CSNP were prepared by ionic gelation method using TPP molecules as cross-linker. The particles morphology and nano size were observed by SEM. The SEM images of the CSNP demonstrate spherical shape (Fig. 4). The size of CSNP ranged between 79.4 and 186.4 nm. Similar results were observed when CSNP were prepared by ionic gelation method, where CSNP with average size of 375 nm [27], 20–60 nm [28], 40–80 nm [21], 50–100 nm [29], 85–115 nm [30]. The ZP of successfully prepared nanoparticles was measured through Malvern, CSNP gave a positive ZP value of +51.9 mV (Fig. 5), the Z-potential distribution has a single peak, indicating excellent uniformity of chitosan nanoparticles. [31] prepared CSNP with ZP of 53.1 mv with chitosan solution of 0.3% concentration.

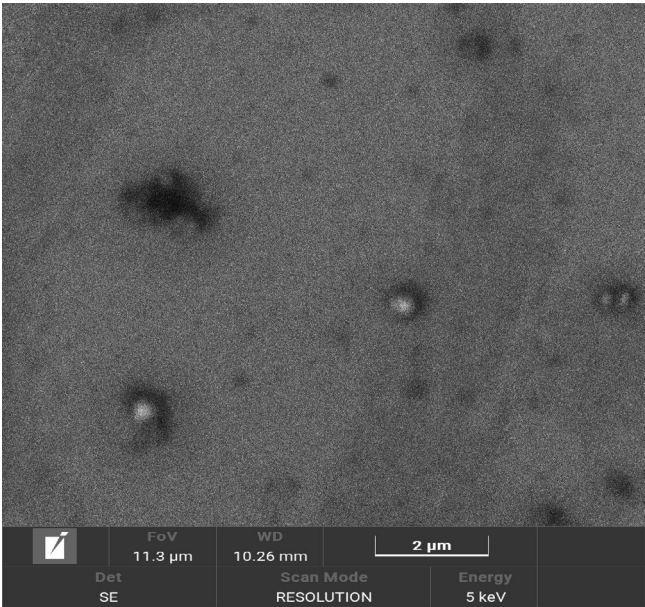


Fig. 4. SEM image of chitosan nanoparticles prepared by ionic gelation method with chitosan solution 1.0%

Source: compiled by E.M. Zeitar, L.T. Sukhenko, S.R. Mohammed.

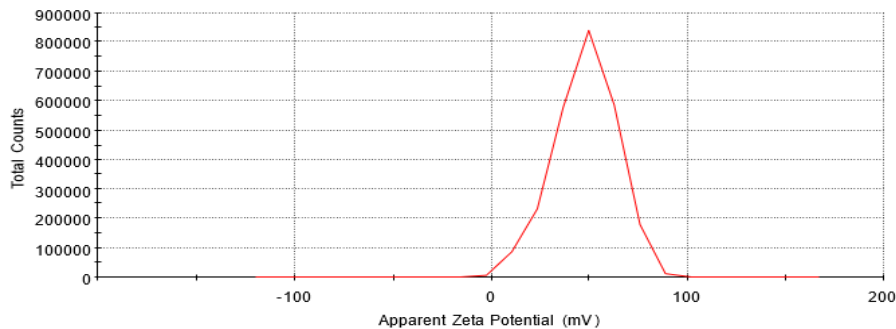


Fig. 5. Zeta potential of CSNP, prepared by ionic gelation method with chitosan solution 1.0%

Source: compiled by E.M. Zeitar, L. T. Sukhenko, S.R. Mohammed.

Antifungal activity on mycelial growth in vitro. Antifungal activity results of Chi, CSNP, and BpE at different concentrations in relation to the mycelial growth on PDA are shown in (Fig. 6). The inhibition of mycelial growth was directly proportional to the concentration of the tested materials. The minimum inhibitory concentrations (MICs) of Chi, CSNP, and BpE were 10.0 g/l, 2.0 g/l, and 40.0 g/l, respectively (Fig. 6). [32] indicated that chitosan at concentration of 1.0% completely inhibited *Rhizoctonia solani* mycelial growth. [31] reported that chitosan nanoparticles at 1500 ppm completely inhibited fungal growth of *Botrytis cinerea*.

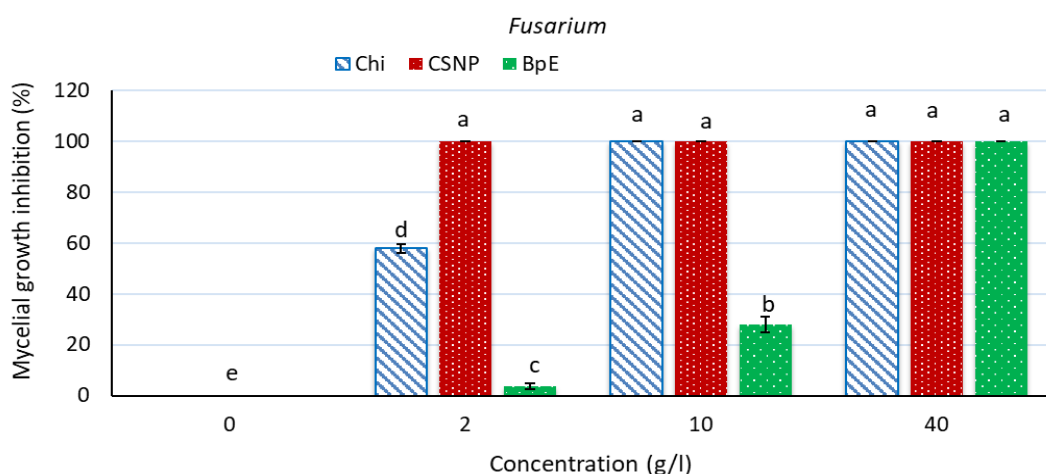


Fig. 6. Antifungal activity of chitosan (Chi), chitosan nanoparticles (CSNP) and black poplar buds extract (BpE) in relation to the mycelial growth of *Fusarium* sp. *in vitro*

Source: compiled by E.M. Zeitar, L.T. Sukhenko, S.R. Mohammed.

Biological efficacy on potato tubers under natural infection. Treatment tubers under a natural infection of *Fusarium* dry rot with CSNP at a rate of 20 g/t and BpE 400.0 g/t significantly reduced the incidence of dry rot by 54.7 and 58.5%, and reduced the disease index by 65.7 and 72.3%, respectively, and there was not a significant difference between the two treatments. The lowest reduction of dry rot incidence was 32.4%, and diseases index 31.4% was observed with treatment by chitosan at a rate of 100 g/t compared with the control (Table 3).

Various factors can influence the biological efficacy of certain components upon interaction with fruit tissue. In agreement with results in our study, [31] reported CSNP showed a gray mould disease severity reduction up to 46.5% on strawberries during storage against *Botrytis cinerea*, and [33] found that CSNP showed 70% disease severity reduction on cucumbers against *Phytophthora drechsleri*.

Table 3

**Effect of chitosan (Chi), chitosan nanoparticles (CSNP)
and black poplar buds extract (BpE) on the disease incidence and index
of dry rot during potato storage under natural infection**

Treatments	Disease Incidence, % (Weight share in the yield, %)	Reduction of Disease Incidence, %	Disease Index (severity), %	Reduction of Disease Index (severity), %
Control	15.7±1.06 (a)	0.0 (d)	10.5±1.03 (a)	0.0 (d)
Chi 100.0 g/t	10.6±0.79 (b)	32.4 (c)	7.2±0.23 (b)	31.4 (c)
CSNP 20.0 g/t	7.1±1.35 (d)	54.7 (b)	3.6±0.82 (c)	65.7 (b)
BpE 400.0 g/t	6.5±0.61(d)	58.5 (b)	2.9±0.53(c)	72.3 (b)
LSD _{0.05}	1.56	7.41	1.34	7.28

Source: compiled by E.M. Zeitar, L.T. Sukhenko, S.R. Mohammed.

Conclusion

In this study, a high virulent *Fusarium* isolate was isolated from natural infected tubers in Astrakhan region and subjected to molecular identification using ITS rDNA gene and identified as *Fusarium sambucinum*. Chitosan nanoparticles were successfully prepared by ionic gelation method with size ranged from 79.4 to 186.4 nm, and a positive zeta potential of 53.1 mv. CSNP at a concentration of 2 g/l and BpE at a concentration of 40 g/l completely inhibited *Fusarium* fungal growth on PDA medium. CSNP at a rate of 20 g/t and BpE at a rate of 400 g/t significantly reduced the incidence of dry rot during 8 months under natural infection at 4 °C by 54.7 and 58.5% and reduced the disease index by 65.7 and 72.3%, respectively, and there were not significant differences between the two treatments. These results demonstrate the potential of CSNP and BpE for protection of potato tubers against the fungal postharvest decay.

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