Entomopathogenic fungus, *Metarhizium anisopliae*, anchored onto MCM-41 silica nanomaterial: A novel and effective insecticide against potato tuber moth in stored potatoes

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Summary *Phthorimaea operculella* (PTM) is an economically significant invasive potato insect pest in tropical and sub-tropical regions. Due to many problems concerning chemical insecticides, biocontrol agents such as entomopathogenic fungi have attracted researchers' interest regarding their application as a part of integrated potato tuber moth management strategies in several countries. Hence, in the present study, we examined the lethal effects of entomopathogenic fungus, *Metarhizium anisopliae* (used as pure culture (PEF) or formulated with MCM-41 silica nanomaterial (MCM-41@fungus)) on eggs and neonate larvae of PTM. The MCM-41 was completely characterized by Fourier transform infrared (FT-IR), Scanning Electron Microscopy (SEM), Energy Dispersive X-ray (EDS), X-ray diffraction (XRD), and Thermogravimetric analysis (TGA) techniques. The morphology of MCM-41@fungus was evaluated by scanning electron microscope (SEM). The morphology of the mesoporous structure was exhibited to be homogeneous, regular, and spherical. LC₅₀ values of PEF and MCM-41@fungus were estimated to be 1.7×10^7 and 2×10^5 conidia/ml for eggs and 1.8×10^6 and 1.5×10^4 conidia/ml for neonate larvae, respectively. Hence, bioassays demonstrated that MCM-41@fungus was more toxic than the pure culture at egg and neonate larval stages of the pest. The results exhibited that pure *M. anisopliae* and its nano-formulation could play key roles as biopesticides in management programmes of *P. operculella*.

Additional keywords: bio-pesticide, MCM-41, *Metarhizium anisopliae*, nano-formulation, *Phthorimaea operculella*, toxicity

Introduction

Potato (*Solanum tuberosum* L. (Solanaceae)) is one of the most important agricultural products that plays a major role as a high value crop for human health and food industry. Among foods, it has the fourth rank in the world after wheat, rice, and corn (Germchi *et al.*, 2011). Potato tuber moth (PTM), *Phthorimaea operculella* Zeller (Lepidoptera: Gelechiidae) is considered as the most damaging potato insect pest in the world (Radcliffe, 1982). PTM occurs in tropical and sub-tropical regions where potato is cultivated

and it causes notable crop yield losses (Kroschel and Sporleder, 2006). Since potato plants resist some PTM damages, protection of stored potatoes against larvae of the pest is more important than in the field (Lacey, 2012). When tubers infested by PTM enter storage without chemical treatment, the larvae continue to develop, make tunnels and pollute the tubers with their frasses and finally the decaying organisms can enter the plant (Radcliffe, 1982).

Extensive use of chemical insecticides has resulted in adverse effects on environment and human health. Particularly, excessive application of non-selective chemical insecticides can destroy natural enemies of the pest and beneficial organisms and induce problems such as development of pest resistance (Sharma and Gupta, 2009). These adverse effects urged the development of alternative pest management tactics in which microbial controls may play principal

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roles (Collantes et al., 1986; Llanderal-Cázares et al., 1996).

Entomopathogenic fungi are efficient microbial control agents of several pests (Gottel et al., 2005), including some key insect pests of potato (Lacey et al., 1999; Wraight and Ramos, 2005). The entomopathogenic filamentous fungus *Metarhizium anisopliae* is a common pathogen of arthropods applied in biological control strategies against numerous serious insect pests (Santi et al., 2010). In spite of the potential of entomopathogenic fungi in pest control, these bio-control agents have some disadvantages including sensitivity to environmental factors such as moisture, light, and temperature that have limited their applications under storage and field conditions. However, these problems can be overcome through recent technological advances such as nano-formulation approaches which can improve their efficacy and pathogenicity and will permit future use of entomopathogenic fungi in crop production systems. In this respect, the hexagonal array of uniform mesoporous MCM-41 with particular properties such as exceptionally high surface areas and high pore volumes (Chen et al., 1993; Rath and Parida, 2011) attracted our interest in applying it in plant protection and insect pest management. Since it has 1-D uniform mesopores, the entomopathogenic fungi can be grafted into MCM-41. Consequently, we selected MCM-41 as a support which belongs to M41S family and is mainly made up of silica, SiO₂, as a carrying agent (Shylesh and Singh, 2005; Rath and Parida, 2011). Silica has unique benefits such as extraordinary thermal and chemical stability, ease of handling, and abundance of exposed silanol (Si-OH) groups (Abdollahi-Ali-beik and Pouriaeyali, 2011).

Materials and Methods

Phthorimaea operculella rearing, *M. anisopliae* culture, nano-formulation

Insect rearing

A colony of potato tuber moth was ob-

tained from the University of Mohaghegh Ardabili, Ardabil, Iran. The colony was continuously reared on Agria potato cultivar. Experiments were carried out under laboratory conditions at $26 \pm 1^\circ\text{C}$, $60 \pm 5\%$ RH and photoperiod of 8:16 (L:D). To achieve cohort eggs of *P. operculella*, 30 male-female pairs of newly emerged moths were kept inside cylindrical containers. The adults were fed using a piece of cotton imbued with a 10% honey-water solution. Cylindrical containers were covered with a fine mesh netting on the heads. Filter papers were placed on the head of containers which provided an oviposition site for moths (Golizadeh and Zalucki, 2012).

Microorganism and culture media

The entomopathogenic fungus, *M. anisopliae* (DS12) was provided from Mycology Collections of Urmia University, Urmia, Iran. This isolate was originally obtained from wheat samples collected from Urmia, Iran. Stock cultures of the isolate were grown on potato dextrose agar slants (PDA; Merck, Germany) and stored at 4°C for future uses. Subcultures were prepared by transferring pieces of stock cultures onto new PDA plates and incubated at $25 \pm 1^\circ\text{C}$ for 14 days before use in experiments. Conidia were harvested by flooding the cultures with sterile distilled water containing 0.02% Tween-80 and scraping with a sterile L-shaped glass rod. Concentrations of the resulting stock suspension were determined using a Neubauer hemocytometer (Fuchs-Rosenthal 0.0025 mm², depth 0.100 mm, VWR, Sweden). Germination was surveyed under a light microscope (400X). A conidium was considered germinated when the germ tube was expanded beyond its width (Inglis et al., 2012). The mean germination rate for *M. anisopliae* was 96%.

Preparation of siliceous MCM-41

Mesoporous Si-MCM-41 was synthesized through Sol-gel method according to Cai et al. (2001).

Preparation of siliceous MCM-41-(CH₂)₃Cl

The resulted MCM-41 powder (4.8g) with 5.0g 3-chloropropyltrimethoxysilane (CPT-

MS) was added to n-hexane (96 ml) and the mixture was stirred under refluxing and nitrogen atmosphere for 24 h. The obtained sediments were collected by filtration, were washed with n-hexane for several times, and eventually dried under vacuum to obtain MCM-41-(CH₂)₃-Cl.

Preparation of MCM-41-entomopathogenic fungus

In a 100ml round bottom flask, a mixture of MCM-41-Cl (1g), entomopathogenic fungus (10ml of determined concentrations) and Et₃N (1.5ml) in H₂O (50ml) were stirred under room temperature for 24h. Then, the final product was dried at room temperature.

Particle morphology studies

Particle morphology was surveyed by a scanning electron microscope (SEM) (Day Petronic Company, Tehran, Iran), using FESEM-TESCAN MIRA3. Thermogravimetric analysis (TGA) curves were recorded on a Shimadzu DTG-60 instrument (University of Kurdistan,

Sanandaj, Iran). Fourier transform infrared (FT-IR) spectra were recorded with KBr pellets on a Nexus 670 FT-IR spectrometer (Medical Sciences of Urmia University, Urmia, Iran). X-ray power diffraction (XRD) patterns were collected on Scintag PAD V X-ray diffractometer using a Co radiation source with wavelength $\lambda = 1.78897 \text{ \AA}$, 40 kV (Shahid Beheshti University, Tehran, Iran). The samples were scanned in the range of $2\theta = 1-10^\circ$.

M. anisopliae was grafted into MCM-41 which led to the synthesis of MCM-41@fungus. The details of MCM-41@fungus preparation procedure are presented in Scheme 1.

Bioassays

Effect on larvae

To examine larval mortality, each potato was dipped in suspensions of pure entomopathogenic fungus and MCM-41@fungus, separately, which were determined by preliminary dose setting experiments. Concentrations of PEF and MCM-41@fungus were

n=fungus structure

Scheme 1. Synthesis of MCM-41@fungus. CPTMS was anchored onto MCM-41 and *Metarhizium anisopliae* was grafted to CPTMS via its hydroxyl groups.

in the ranges of 10^4 - 10^8 and 10^2 - 10^6 conidia/ml, respectively. In MCM-41@fungus treatments, 0.1g of each nano-composite was dispersed in 100ml distilled water containing 0.02% Tween-80 until water absorbance was stabilized. After shaking and product dispersion, potato tubers were dipped in the corresponding solutions for 15s. When water was evaporated and tubers were dried, potato tubers were transferred into plastic containers with ventilated lids that were kept at $26 \pm 1^\circ\text{C}$, $60 \pm 5\%$ RH and a photoperiod of 8 L:16 D. Then, 20 newly emerged larvae (<5 h old) were placed on each tuber using a soft hair brush. Criterion of larval penetration was the number of adult emergence in all experiments. Each treatment and dose level (5 concentrations) was replicated three times.

Effect on egg hatching

PEF and MCM-41@fungus were tested at 10^5 - 10^9 and 10^3 - 10^7 conidia/ml, respectively. For treatments, filter papers containing 20 one-day-old eggs/each concentration were separately dipped in solutions of PEF and MCM-41@fungus. Preparation of MCM-41@fungus treatments was carried out in a similar way to the previous one. When filter papers dried, they were transferred into plastic containers with ventilated lids containing intact potato tubers to stimulate egg hatching. The containers were kept under laboratory conditions ($26 \pm 1^\circ\text{C}$, $60 \pm 5\%$ RH and a photoperiod of 8L: 16D). Egg hatch was investigated using a light microscope after eight days. Each treatment was replicated three times.

Statistical analysis

In order to determine LC_{50} values, the data were analyzed utilizing probit procedures with software SPSS for Windows® version 16.

Results and Discussion

Characterization of MCM-41 substrate and its use as a carrying agent in nano-

formulation of *M. anisopliae*

Fungus was supported on mesoporous MCM-41 which was completely characterized by Fourier transform infrared (FT-IR), Scanning Electron Microscopy (SEM), Energy Dispersive X-ray (EDS), X-ray diffraction (XRD), and Thermogravimetric analysis (TGA) techniques.

Fourier transform infrared (FT-IR) spectra, recorded separately at different steps of MCM-41@fungus synthesis, can be observed in curves a-b of Figure 1. As presented in curve a, for MCM-41 sample, the absorption bands at 1230, 1058.07, 812.10, and 463.30 cm^{-1} were assigned to asymmetric and symmetric stretching vibrations of mesoporous framework (Si-O-Si). The band at 3431.91 cm^{-1} was attributed to the stretching vibration of O-H groups. Curve b demonstrated stretching vibrations at 1520.45 (C-N), and 1690.89 cm^{-1} (C=O) and a broad band at around 3440.07 cm^{-1} (O-H and N-H). The band at 1375.95 cm^{-1} corresponded to the bending vibration of CH_3 group, and the presence of the anchored CPTMS group was confirmed by C-H stretching vibrations at 2950 and 2865 cm^{-1} for MCM-41@fungus.

The Energy Dispersive X-ray (EDS) of mesoporous MCM-41@fungus displays the grafting of fungus onto MCM-41 surface (Fig. 1). The results showed that there were only C, Si, O, and N elements in the nano-pores with relative mass percentages of 15.50, 48.81, 32.15, and 3.54%, respectively (Fig. 2). Scanning electron microscope (SEM) micrograph of mesoporous MCM-41@fungus is demonstrated in Figure 3, showing that the particles were in nano range (<100 nm). The morphology of the mesoporous MCM-41@fungus was homogeneous, regular, and spherical. Figure 4 demonstrates slow angle X-ray powder diffraction pattern of the samples (MCM-41 and MCM-41@fungus). XRD pattern of MCM-41 displayed three reflection peaks corresponding to d100 plane (very strong reflection) at $2\theta=2.37^\circ$ and two peaks at d110 and d200 planes (weaker reflections) at $2\theta=4.3^\circ$ and 4.48° , respectively, which can be attributed to the hexagonal structure of MCM-41. The values of spacing

Figure 1. (a) Fourier transform infrared (FT-IR) spectra of MCM-41 and (b) MCM-41@fungus of the entomopathogenic fungus *Metarhizium anisopliae*.

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Figure 2. Energy Dispersive X-ray (EDS) spectrum of MCM-41@fungus of the entomopathogenic fungus *Metarhizium anisopliae*: There were only C, Si, O, and N elements in the nano pores with relative mass percentages of 15.50, 48.81, 32.15, and 3.54%, respectively.

(100) for XRD patterns of MCM-41 and MCM-41@fungus were 37.14 and 32.98 $\text{\AA}$, respectively. As demonstrated, after functionalization steps, a considerable decrease in XRD peak intensities was observed which caused variations in wall thickness and might be due to the reduction of scattering contrast between the channel wall of silicate framework and fungus. Therefore, it can be concluded that the grafting of fungus occurred

Figure 3. SEM image of MCM-41@fungus mesoporous of the entomopathogenic fungus *Metarhizium anisopliae*: The particles are observed to be in the nano range (<100 nm). The mesoporous presents homogeneous, regular, and spherical morphology.

Figure 4. (a) X-ray diffraction (XRD) patterns of MCM-41 and (b) MCM-41@fungus of the entomopathogenic fungus *Metarhizium anisopliae*: The values of spacing (100) for XRD patterns of MCM-41 and MCM-41@fungus were 37.14 and 32.98 $\text{\AA}$, respectively. As demonstrated, after functionalization steps, a considerable decrease in XRD peak intensities was also observed.

in the inner pore channels of Si-MCM-41.

Thermogravimetric analysis (TGA) was employed to select the weight changes of functionalized mesoporous MCM-41. TGA curves for MCM-41(a) and MCM-41@fungus (b) are depicted in Figure 5. According to the TGA curve b, an initial weight loss was seen at temperatures below 200 $^{\circ}\text{C}$ because of the removal of physically and chemically adsorbed water molecules inside the pores

channels and surface hydroxyl groups. Additionally, at temperatures above 200°C, large weight losses occurred which were mainly due to the decomposition of covalently bonded organics (200-500°C) and silanol groups (>500°). This shows that the grafting of fungus had occurred on the inner pore channels of Si-MCM-41. MCM-41 has attracted great attention because of its large pore size and extremely high surface area especially above 1000 m²/g (Nikoora-zm et al., 2015). The exceptionally high surface area and porous structure of MCM-41 make it as a potential efficient host material for a variety of supported catalysts (Nikoora-zm et al., 2015). According to our results,

Figure 5. (a) TGA thermograms of MCM-41(a) and (b) MCM-41@fungus of the entomopathogenic fungus *Metarhizium anisopliae*: At temperatures above 200°C, large weight losses occurred which were mainly due to the decomposition of covalently bonded organics (200-500°C) and silanol groups (>500°), indicating grafting of fungus on the inner pore channels of Si-MCM-41.

the entomopathogenic fungus *M. anisopliae* was grafted to MCM-41 through the hydroxyl group of the fungus leading to the synthesis of MCM-41@fungus.

MCM-41@fungus against eggs and neonate larvae of *P. operculella*

The results of the bioassays for PEF and MCM-41@fungus against eggs and neonate larval penetration of PTM are presented in Table 1. LC₅₀ values of PEF and MCM-41@fungus were 1.7×10⁷ and 2.0×10⁵ conidia/ml on eggs and 1.8×10⁶ and 1.5×10⁴ conidia/ml on neonate larvae, respectively. Hence, at both life stages, LC₅₀ values of nano-formulated entomopathogenic fungus against *P. operculella* were significantly lower thus more effective than pure *M. anisopliae*, as indicated by the absence of overlapping of confidence limits. Sabbour (2014) also found similar results on the efficacy of nano-formulated destruxin of *M. anisopliae* against adult females of *Heteracris littoralis* Rambur (Orthoptera: Acrididae) under laboratory conditions, i.e., in nano-formulated samples, LC₅₀ values were significantly decreased.

Susceptibility of potato tuber moth larvae to *Baeuveria bassiana* and *M. anisopliae* and the role of chemical additives in enhancing the action of entomopathogenic fungi were assessed by Sabbour (2002) who noted that oxalic and citric acids demonstrated the highest improvement of *M. anisopliae* efficacy against first instar lar-

Table 1. Toxicity of *Metarhizium anisopliae* and MCM-41@fungus to eggs and first instar larvae of *Phthorimaea operculella* under laboratory conditions.

Growth stages	Treatments	Slope ± S. E.	χ ² (df)	LC ₅₀ (conidia/ml)	LC ₉₀ (conidia/ml)
Egg	PEF	3.74 ± 0.05	1.47 (3)	1.7 × 10 ⁷ (6.3 × 10 ⁶ -5.2 × 10 ⁷)	9.2 × 10 ¹⁰ (8.9 × 10 ⁹ -6.9 × 10 ¹²)
	MCM-41@fungus	4.26 ± 0.08	1.26 (3)	2.0 × 10 ⁵ (7.1 × 10 ⁴ -6.6 × 10 ⁵)	1.5 × 10 ⁹ (1.2 × 10 ⁸ - 1.9 × 10 ¹¹)
Neonate larvae	PEF	3.94 ± 0.06	1.00 (3)	1.8 × 10 ⁶ (6.6 × 10 ⁵ - 6.1 × 10 ⁶)	1.4 × 10 ¹⁰ (1.2 × 10 ⁹ - 1.7 × 10 ¹²)
	MCM-41@fungus	4.57 ± 0.09	1.63 (3)	1.5 × 10 ⁴ (5.3 × 10 ³ -5.0 × 10 ⁴)	1.4 × 10 ⁸ (1.1 × 10 ⁸ -1.8 × 10 ¹⁰)

PEF: Pure Entomopathogenic Fungus (=non-formulated fungus)
95% fiducial limit (FL) is shown in parenthesis

vae of *P. operculella* (LC_{50} values = 1.20×10^7 and 1.30×10^7 conidia/ml, respectively, compared with $LC_{50} = 8.61 \times 10^7$ conidia/ml for *M. anisopliae* alone). All LC_{50} values were higher than the LC_{50} value of PEF obtained from our results on larvae, possibly due to variations in the isolates and experimental procedures, however the chemical additives increased the efficiency of *M. anisopliae* against the pest. On the other hand, Khorrami *et al.* (2018) found a lower LC_{50} value (= 1.9×10^5 conidia/ml) relatively to our results for pure *M. anisopliae*, which could be also attributed to variation in the entomopathogenic fungus isolate. Other studies have also revealed the efficacy of *M. anisopliae* in pest control (Lacey *et al.*, 1994; Ansari *et al.*, 2004, 2007; Marannino *et al.*, 2006; Meyling and Eilenberg, 2007; Baker *et al.*, 2018) while Khorrami *et al.* (2018) evaluated the pathogenicity of *M. anisopliae* against the potato tuber moth in comparison with *Nomuraea rileyi* and *Paecilomyces tenuipes*. In the latter study, *N. rileyi* presented the highest toxicity against neonate larvae of PTM in laboratory conditions (LC_{50} value equivalent to 1.0×10^3 conidia/ml) whereas *M. anisopliae* and *P. tenuipes* had lower activity (LC_{50} values equivalent to 1.9×10^5 and 2.4×10^6 conidia/ml, respectively).

Based on our findings on the effect of pure *M. anisopliae* and MCM-41@fungus, neonate larvae are more susceptible to infection by *M. anisopliae* than its eggs, which is consistent with the conclusions of Khorrami *et al.* (2018). Similarly, Sewify *et al.* (2000) reported that *M. anisopliae* has a potential for control of potato tuber moth larvae. They also reported that the combination of *M. anisopliae* and phoGV had a synergistic effect for larval control. Pandey *et al.* (2015) examined efficacy of *M. anisopliae* against PTM under laboratory conditions and exhibited that the fungus showed high biological activity against *P. operculella* larvae with LC_{50} values, ranging from 2.94×10^5 to 7.16×10^7 conidia/ml. In addition, Marannino *et al.* (2006) found that *M. anisopliae* isolates were more virulent to neonate larvae of *Capnodis tenebrionis* Eschscholtz than the

eggs of the pest. The results are promising since the first larval stage is the only free-living stage exhibiting a potential practical target in storage. To our knowledge, MCM-41 has not been used as carrying agent in bio-pesticides and this is the first report of using of MCM-41 as carrying agent that can significantly improve the effectiveness of the *M. anisopliae* on eggs and neonate larvae of *P. operculella*. The experimental protocol followed in the present study allowed us to provide evidence for MCM-41@entomopathogenic fungus infection.

Conclusion

Laboratory bioassays have clearly demonstrated the pathogenicity of *M. anisopliae* and MCM-41@*M. anisopliae* for neonate larvae and eggs of *P. operculella*. These bio-control agents should be considered for the development of a new and environmentally compatible approach for potato tuber moth management in terms of preventing PTM infestations. Further research is required to determine the biological activities of MCM-41@*M. anisopliae* and its persistence after store applications and other factors that may improve its performance against *P. operculella* throughout its egg and larval stages.

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Σκεύασμα του εντομοπαθογόνου μύκητα *Metarhizium anisopliae* με νανοϋλικά πυριτίου MCM-41: Ένα νέο και αποτελεσματικό εντομοκτόνο κατά της φθοριμαίας της πατάτας σε αποθηκευμένους κονδύλους

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Περίληψη Η φθοριμαία της πατάτας, *Phthorimaea operculella*, αποτελεί οικονομικής σημασίας εντομολογικό εχθρό της πατάτας σε τροπικές και υποτροπικές περιοχές. Λόγω πολλών προβλημάτων σε σχέση με τη χημική καταπολέμηση του εντόμου, οι παράγοντες βιολογικής αντιμετώπισης, όπως οι εντομοπαθογόνοι μύκητες, έχουν προσελκύσει το ενδιαφέρον των ερευνητών σχετικά με την εφαρμογή τους ως μέρος ολοκληρωμένων στρατηγικών διαχείρισης του εντόμου σε πολλές χώρες. Ως εκ τούτου, στην παρούσα εργασία εξετάστηκε η εντομοκτόνος δράση του εντομοπαθογόνου μύκητα *Metarhizium anisopliae* (ως καθαρή καλλιέργεια ή σε σκεύασμα με νανοϋλικά πυριτίου MCM-41 (MCM-41@fungus)) σε ώα και νεοεκκολαφθείσες προνύμφες του εντόμου. Το νανοϋλικό MCM-41 χαρακτηρίστηκε πλήρως με τεχνικές Φασματοσκοπίας Υπερύθρου Μετασχηματισμού Fourier (FT-IR), Ηλεκτρονική Μικροσκοπία Σάρωσης (SEM), Ακτίνες Χ Διασποράς Ενέργειας (EDS), Περίθλαση ακτίνων Χ (XRD) και Θερμοβαρυμετρική ανάλυση (TGA). Η μορφολογία του νανο-σκευάσματος MCM-41@fungus αξιολογήθηκε με ηλεκτρονικό μικροσκόπιο σάρωσης (SEM). Η μορφολογία της μεσοπορώδους δομής του βρέθηκε ότι είναι ομοιογενής, κανονική και σφαιρική. Οι τιμές LC_{50} της καθαρής καλλιέργειας του μύκητα και του νανο-σκευάσματος MCM-41@fungus υπολογίστηκαν ότι είναι $1,7 \times 10^7$ και 2×10^5 κονίδια/ml για τα ώα και $1,8 \times 10^6$ και $1,5 \times 10^4$ κονίδια/ml για τις νεοεκκολαφθείσες προνύμφες, αντίστοιχα. Επομένως, οι βιοδοκιμές έδειξαν ότι το σκεύασμα MCM-41@fungus ήταν πιο δραστικό από την καθαρή καλλιέργεια έναντι και των δύο σταδίων του εντόμου. Η καθαρή καλλιέργεια του *M. anisopliae* και το νανο-σκεύασμα αυτού θα μπορούσαν να αξιοποιηθούν ως βιο-εντομοκτόνα σε προγράμματα διαχείρισης της φθοριμαίας της πατάτας.

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