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Fungi Isolated from Spoiled Bean Sprouts in Japan

Toyozo SATO[1]*, Mutsuo AOKI[2], Takayuki AOKI[1], Masaharu KUBOTA[3], Takashi YAGUCHI[4], Shihomi UZUHASHI[5] and Keisuke TOMIOKA[6]

[1] Genetic Resources Center, National Institute of Agrobiological Sciences (Tsukuba, Ibaraki 305-8602, Japan)
[2] Sun-Tommy International Co. Ltd., (Chuo, Tokyo 103-0027, Japan)
[3] Institute of Vegetable and Tea Science, National Agriculture and Food Research Organization (NARO) (Tsukuba, Ibaraki 305-8666, Japan)
[4] Medical Mycology Research Center, Chiba University (Chiba, Chiba 260-8673, Japan)
[5] Microbe Division, Japan Collection of Microorganisms, RIKEN BioResource Center (Tsukuba, Ibaraki 305-0074, Japan)
[6] Western Region Agricultural Research Center, NARO (Fukuyama, Hiroshima 721-8514, Japan)

Abstract

The morphology of 18 and seven species of fungi isolated from spoiled bean sprouts of *Vigna* spp. and soybean in Japan were respectively described, and DNA barcode markers of most isolates were sequenced to confirm the morphological identification. Fifteen and five species were isolated for the first time from *Vigna* spp. and soybean sprouts, including their ingredient grains, respectively. *Globisporangium ultimum* var. *ultimum* was newly recorded from the mung bean and most isolated fungi seemed to originate from the grains. Approximately 70% of isolates are recognized as plant pathogens and at least 14 species are known to be seed-borne. Inoculation experiments with representative strains of each species are needed to estimate the risks to bean sprout production and crop protection. Some strains of *Fusarium graminearum* isolated from the soybean were already reported as producing high concentrations of deoxynivalenol. *Aspergillus flavus*, which was found in mung bean sprouts, is a well-known aflatoxin producer. The ingredient grains should be imported after complete sterilization to avoid hazards; not only to bean sprout production but to human health. The effects of previously used sterilization techniques should be re-examined with the strains of various fungi isolated in this study to make them more practical.

Discipline: Food
Additional key words: black matpe, fungal carrier, identification, mung bean, soybean

Introduction

Bean sprouts, mainly made from grains of the mung bean (green gram, *Vigna radiata* (L.) Wilczek.), black matpe (black gram, *Vigna mungo* (L.) Hepper.) or soybean (*Glycine max* (L.) Merr.), are common raw foods in Eastern Asia including Japan. They are often used year-round in Chinese, Korean and Japanese cooking as an inexpensive vegetable. More than 380,000 t of mung bean sprouts were produced by 150 processors in Japan in 2008 (Castamhouse of Okinawa district, 2010). Recently, all the ingredient grains have been imported to Japan from Asian countries. For example, 55,534 t (around 90%) of mung bean and black matpe were imported from China, and imports of the remaining 6,258 t (around 10%) were shared by Myanmar, Thailand, Malaysia and India in 2009 (Castamhouse of Okinawa district, 2010). Conversely, a few thousand tons of soybeans have been imported every year mainly from China and the United States (Aoki et al., 2000).

The grains are incubated at warm temperatures, 25-30°C, with sufficient water and humidity during the bean sprout processing. These conditions also favor the propagation of various microorganisms, including plant pathogenic bacteria and fungi. A rot of mung bean sprouts caused by *Cylindrocephalum* sp. was reported in the United States (Cody and Maloy, 1984). Aoki et al. (1986) first detected two species of bacteria, *Erwinia carotovora*

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*Corresponding author: e-mail s1043@affrc.go.jp
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(=*Pectobacterium carotovorum*) and *Pseudomonas fluorescens* Biotype II, and four fungi, *Colletotrichum* sp., *Fusarium solani* (Mart.) Sacc., *Macrophomina phaseolina* (Tassi) Goid. and *Rhizoctonia solani* J. G. Kühn, spoiling bean sprouts of *Vigna* spp. in Japan. *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., *Fusarium oxysporum* Schltdl., *F. solani*, *M. phaseolina* and *Rhizopus oryzae* Went & Prins. Geerl. isolated from *Vigna* spp., *Alternaria alternata* (Fr.) Keissl. and *Fusarium graminearum* Schwabe from soybean were subsequently subjected to microwave sterilization tests together with steam (Aoki et al., 2000). *Cercospora kikuchii* (Tak. Matsumoto & Tomoy.) M. W. Gardner, which was listed in this report as a fungus spoiling soybean sprouts, was not examined using the sterilizing technique (Aoki et al., 2000). *Alternaria alternata*, *C. gloeosporioides* and *F. oxysporum* were also used to estimate the antimicrobial effects of allylisothiocyanate (Furuya et al., 2002). *Fusarium moniliforme* J. Sheld. [*Gibberella fujikuroi* (Sawada) Wollenw.], *F. oxysporum*, *F. solani* and *Pythium deliense* Meurs were identified as causes of soybean sprouts rot in Korea (Oh and Park, 1996; Yun and Kim, 2003). However, mycological identification of the fungal species previously reported from Japan have never been demonstrated and other unknown fungi have also been isolated from spoiled or rotted bean sprouts. In this paper, the morphology of fungi isolated from spoiled bean sprouts of *Vigna* spp. and soybean are described, and DNA barcode markers were sequenced to confirm the morphological identification. We expected risks in bean sprout production, crop protection and human health based on the fungi isolated and their previously reported properties.

Materials and Methods

1. Damage characteristics of bean sprouts and origin of ingredient grains

Spoiling or rotting characteristics of bean sprouts were observed and photographed. The location in which the spoiling or rotting was found and the country from which the ingredient grains were imported were recorded.

2. Morphological identification of fungi isolated

Single-hyphal or single spore isolates were obtained by incubating contaminated grain and spoiled or rotted sprouts on potato dextrose agar (PDA, Difco Laboratories, Detroit, MI, USA) plates after surface sterilizing by soaking in 70% ethanol for 10 s and subsequently in 1% sodium hypochloride for 1 min. Representative isolates were deposited into the NIAS Genebank, National Institute of Agrobiological Sciences, Japan. Mycelial discs (around 6 mm in diameter) of strains with MAFF accessions listed in Tables 1 and 2 were transferred onto PDA plates, then incubated at 25°C in the dark for 7-14 days to observe and take photos of their colonies. Synthetic low nutrient agar medium (SNA, Aoki & O'Donnell, 1999) amended with autoclaved filter paper pieces and PDA were used for microscopic observation of *Fusarium* species and others, respectively. Small agar pieces containing mycelia from PDA slant cultures of the strains listed in Tables 1 and 2 were transferred to SNA or PDA plates (90 mm in diam.). After 7-14 days of incubation at 25°C in the dark, strains bearing no fruiting bodies were placed under a black light (Toshiba FL20SBLB, peak emission 352 nm) at a distance of around 20 cm and re-incubated at 25°C. Anastomosis and homogenous groups (AG and HG) of two *R. solani* strains were determined according to the procedure by Hagiwara et al. (2008). The length and width of 30 to 50 reproductive organs of each were measured with differential interference contrast illumination (Nikon Eclipse 80i with an image analyzer, Nikon Digital Sight, Nikon, Tokyo, Japan), while the organs of the strains were photographed with a digital camera attached to the microscope and scanning electron micrographs were taken with low vacuum-type SEM (Keyence VE-7800, Keyence, Tokyo, Japan) without pretreatment. The strains examined were identified based on morphological comparisons with those described in the References listed in Tables 1 and 2.

3. DNA sequencing and BLASTN search

The internal transcribed spacer (ITS) region, including the 5.8S rRNA gene of most isolated strains (Tables 1, 2), were sequenced. Partial sequences of the β-tubulin-2 gene (TUB2) were obtained for strains belonging to the genera *Colletotrichum*, *Aspergillus* and *Penicillium*. Genomic DNA was extracted and both loci were sequenced according to the protocol by Sato & Moriwaki (2013). The partial histone H3 gene (HIS3) of two *Fusarium* strains was sequenced as described by O'Donnell et al. (2004). All of the sequences were published from the web pages, "Detailed information of microorganism genetic resources of Microorganism Search System", NIAS Genebank, Japan (http://www.gene.affrc.go.jp/databases-micro_search_en.php) or DDBJ/EMBL/GenBank databases. Accession numbers of sequences are listed in Tables 1 and 2 in the latter case. The sequence data were searched with "Standard Nucleotide BLAST" in the NCBI website (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PAGE=MegaBlast&PROGRAM=blastn&BLAST _PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&BLAST_SPEC=) to confirm the results of morphological identification. Representative accession(s) and a corresponding fungal name with 99-100% identity hit in each BLASTN search are listed in Tables 1 and 2.

Table 1. Fungi isolated from contaminated grains or spoiled sprouts of the mung bean (*Vigna radiata*) and black matpe (*V. mungo*)

Division[a]	Species[b]	Isolation source[d]	Isolation location	Producing country	Year	MAFF accession[e]	DNA sequence[f]	BLASTn hit (accession)	Homology[g]	Hit species	Reference[h]
A	*Alternaria alternata**[S]	G	Tokyo	China	1986	239884	I	HQ263343	100/100	*A. alternata*	SR
		G	Tokyo	China	1998	239887	I	HQ263343	100/100	*A. alternata*	SR
A	*Alternaria* sp.	G	Tokyo	China	2012	243775	n				KM
A	***Arthrinium arundinis***	S	Miyagi	China	1997	238039	I	KF144888	100/100	*A. arundinis*	C, G
A	***Aspergillus flavus****	S	Tokyo	China	2012	243495	T (AB849500)	JX627689	100/100	*A. flavus*	SR
A	***Aspergillus niger****	R	Miyazaki	Thailand	2005	239777	n				SR
		S	Tokyo	China	2012	243496	n				
A	***Chaetomium* sp.**[S]	G	Tokyo	China	2012	243477	n				Ha
A	***Colletotrichum chlorophyti*****[S]	S	Tokyo	China	1984	305748	T	GU228189	99/66	*C. chlorophyti*	D
							I	JX126475	99/94		
		S	Tokyo	China	1988	306157	T	AB697050	100/100	*C. nymphaeae*	SM
							I	AB618089	100/96		
A	***Colletotrichum nymphaeae****[S]	S	Tokyo	China	1992	306343, 306344	T	AB697050	100/100	*C. nymphaeae*	
							I	AB618089	100/96		
		S	Chiba	China	2009	242581	T	AB697050	100/100	*C. nymphaeae*	
							I	AB618089	100/100		
A	***Fusarium equiseti***	R	Tokyo	Thailand	2002	239547, 239918	n				SR
A	*Fusarium oxysporum**[S]	S	Chiba	China	1998	238041	H	AF150832	100/100	*F. oxysporum*	SR
		G	Niigata	China	2005	239895	n				
A	*Fusarium* sp.	S	Tokyo	Myanmar	2013	244040	n				KM
A	***Geotrichum candidum*** (*Galactomyces geotrichum*)*	S	Miyagi	China	1997	238038	I	JQ668740	100/100	*G. geotrichum*	
		G	Miyagi	China	1997	239885	I	KC143429	99/100	*Galactomyces* sp.	SR
		S	Niigata	China	2004	239771	I	JQ668739	99/100	*G. geotrichum*	
		S	Tokyo	China	1984	305746	I	FJ395247	100/100	*M. phaseolina*	Ho
A	*Macrophomina phaseolina**[S]	G	Tokyo	China	1986	239878	I	FJ415067	99/98	*M. phaseolina*	
		S	Niigata	China	2005	239772, 239898	I	FJ415067	99/98	*M. phaseolina*	
		G	Niigata	China	2005	239894	I	FJ415067	99/98	*M. phaseolina*	
A	***Phoma* sp.**	G	Tokyo	China	2012	243774	n				KM
A	***Phomopsis phaseoli* var. *phaseoli*** (*Diaporthe phaseolorum* var. *phaseolorum*)*[S]	G	Tokyo	Myanmar	2011	242916, (242917)	I	JF896458	99/97	*Diaporthe phaseolorum*	K, G
A	***Phomopsis* sp.** (*Diaporthe* sp.)	S	Tokyo	China	2012	243678	n				KM
A	***Trichoderma* sp.**	G	Tokyo	Canada	2011	242918	n				SR
B	*Rhizoctonia solani* (*Thanatephorus cucumeris*)*	S	Tokyo	China	1984	305749	n				
B	*R. solani* (**AG-4, HG-I**)[c]*	S	Niigata	China	2005	239817	I	EU591781	99/100	*R. solani*	JJ
B	*R. solani* (**AG-4, HG-III**)[c]*	G	Tokyo	China	2008	241477	I	DQ102449	99/100	*T. cucumeris*	
O	***Globisporangium ultimum* var. *ultimum*** (*Pythium ultimum* var. *ultimum*)*	G	Tokyo	China	2008	241478	I				P, U
Z	***Lichtheimia ramosa*** (*Absidia corymbifera* var. *ramosa*)	S	Tokyo	China	1992	306273	I	FJ719373	100/98	*L. ramosa*	SR
Z	***Rhizopus oryzae****[S]	S	Tokyo	China	1997	238040	I	AY213684	100/98	*R. oryzae*	SR
		G	Miyagi	China	1997	239882	I	AY213684	100/98	*R. oryzae*	

[a] A:Ascomycota, B: Basidiomyccota, O: Oomyccota, Z: Zygomyccota

[b] bold: new records for the Vigna bean sprout, *: reported as a plant pathogen in Japan (Anonymous, 2012), **: reported as a plant pathogen abroad (Damm et al., 2009), S: reported as seed-borne (Malone et al., 1997)

[c] AG: anastomosis group, HG: homogeneous group

[d] G: grain of *Vignag radiata*, S: sprout of V. *radiata*, R: sprout of *Vigna mungo*

[e] Strains preserved in the NIAS Genebank, National Institute of Agrobiological Sciences, Japan

[f] I: ITS region, T: β-tubulin-2, H: HistoneH3, n: not sequenced, ( ): accession of DDBJ/EMBL/GenBank

[g] identity (%)/query coverage (%)

[h] C: Crous & Groenewald (2013), D: Damm et al. (2009), G: Gomes et al. (2013), Ha: Hanlin (1990), Ho: Holliday (1980), JJ: Johnk & Jones (2001), K: Kulik (1984), KM: Kiffer & Morelet (2000), P: Plaats-Niterink (1981), SM: Sato & Moriwaki (2013), SR: Samson & Reene-Hoekstra (1988), U: Uzuhashi et al. (2010)

Table 2. Fungi isolated from contaminated grains or spoiled sprouts of the soybean (*Glycine max*)

Division[a)]	Species[b)]	Isolation source[c)]	Isolation location	Producing country	Year	MAFF accession[d)]	DNA sequence[f)]	BLASTn hit (accession)	Homology[g)]	Hit species	Reference[h)]
A	*Cercospora kikuchii**[S]	G	Tokyo	China	1986	239883	I	JX143619, HM631726	99/97	*C. kikuchii*	HG
A	***Diaporthe phaseolorum* var. *caulivora*****[S]	G	Tokyo	USA	2009	none[e)]	I	KC343046	100/100	*D. caulivora*	K, G
A	*Fusarium graminearum* (*Gibberella zeae*)*[S]	G	Tokyo	USA	2008	241713-241717	I	DQ459827	100/99	*G. zeae*	SR, Ha
		S	Tokyo	China	1998	238042	H	AY452853	100/100	*G. zeae*	
		G	Tokyo	China	2012	243773	n				
A	*Fusarium oxysporum**[S]	G	Tokyo	China	1998	239879	n				SR
		S	Ibaraki	China	2009	242580	I	KC196121	100/100	*F. oxysporum*	
A	***Penicillium oxalicum***	S	Ibaraki	China	2009	242579	I, T (AB849501)	JQ446378 KC344992	100/98 99/99	*P. oxalicum*	SR
A	***Phoma medicaginis****[S]	G	Tokyo	China	2004	239889	I	HQ630963	99/100	*Phoma* sp.	B
		G	Tokyo	China	2007	240348	I	EU273521	99/100	*Phoma* sp.	
A	***Phomopsis phaseoli* var. *sojae*** (*Diaporthe phaseolorum* var. *sojae*)*[S]	S	Tokyo	China	2012	243679	n				K, G
Z	***Syncephalastrum racemosum***	S	Tokyo	China	2009	241792	n				SR

[a)] A: Ascomycota, Z: Zygomycota

[b)] bold: new records to soybean sprout, *: reported as a plant pathogen in Japan (Anonymous, 2012), **: reported as a plant pathogen abroad (Kulik, 1989; Hartman et al., 1999), [S]: reported as seed-borne (Malone et al., 1997; Hartman et al., 1999),

[c)] G: grain, S: sprout

[d)] Strains preserved in the NIAS Genebank, National Institute of Agrobiological Sciences, Japan

[e)] Gas-sterilized and dried culture specimen

[f)] I: ITS region, T: β-tubulin-2, H: HistoneH3, n: not sequenced, ( ): accession of DDBJ/EMBL/GenBank

[g)] identity (%)/query coverage (%)

[h)] B: Boerema et al. (2004), G: Gomes et al. (2013), Ha: Hanlin (1990), HG: Hsieh & Goh (1990), K: Kulik (1984), SR: Samson & Reene-Hoekstra (1988)

Results

1. Damage characteristics of bean sprouts and origin of ingredient grains

Spoiling or rotting of mung bean sprouts is shown in Figs. 1A, 1F, 1T, 1Y, 2G, 2M, 2R, 3H, 3P and 3U. The ingredient grains in the photos were all imported from China. Fig. 2A shows girdling rot of black matpe imported from Thailand. Damaged soybeans are displayed in Figs. 4A, 4J, 4S and 4V. The ingredient grains in the photos were all imported from China except for that in Fig. 4J, which was introduced from the United States for germination tests by a material supplier. The characteristics of the spoiling or rotting are described in connection with the fungi isolated in the following section.

2. Fungi isolated from *Vigna* spp. bean sprouts

At least 37 fungal isolates identified as 18 species of 15 genera were obtained from the mung bean (*V. radiata*) or black matpe (*V. mungo*). The morphology of each species is described below, and the results of the BLASTn search are summarized in Table 1.

***Alternaria alternata* (Fr.) Keissl.** was isolated twice from rotted roots that were dark brown to black in color (Fig. 1A). Colonies: Surface fluffy, grayish olive to brown, reverse darker than the surface (Figs. 1B, C). Conidia: Chained, obclavate, obovoid, with transverse and often oblique or longitudinal septa, pale brown to brown, minutely berrucose, 22-87 × 8-13.5 (av. 52.4 × 11.0) µm, beaked (3-35.6 (av. 14.4) µm long) (Fig. 1D).

***Alternaria* sp.** Conidia: Similar to those of *A. alternata*, 11.5-31.6 × 7-13.6 (av. 21.3 × 10.5) µm, beaked (3.8-9.4 (av. 6.4) µm long) (Fig. 1E).

***Arthrinium arundinis* (Corda) Dyko & B. Sutton** was isolated from light-brown hypocotyl and roots covered with white hyphae (Fig. 1F). Colonies: Surface with sparse and white aerial mycelia, reverse cream to pale yellow (Figs. 1G, H). Conidiophores: Cylindrical, hyaline, smooth, 0.5 µm wide, 4-14 (-34) µm long. Conidia: Aseptate, lenticular, smooth, dark brown with clear margin, 4.8-7.2 × 3.6-4.6 (av. 5.8 × 4.0) µm (Figs. 1I, J).

***Aspergillus flavus* Link** Colonies: Surface powdery, yellowish-green (Fig. 1K). Conidial heads: With radiate phialides. Conidiophores: Cylindrical, rough, hyaline, 12 µm wide (Figs. 1L, M). Conidia: Aseptate, globose to subglobose, pale yellow, 3.4-4.8 (av. 4.1) µm in diam. (Fig. 1M).

***Aspergillus niger* Tiegh.** Colonies: Surface powdery, dark brown to black (Fig. 1N). Conidiophores: Long cylindrical, hyaline, smooth, 14 µm wide, more than 200 µm long, conidial heads were subglobose, 47-90 µm in diam. Conidia: Aseptate, globose, minutely berrucose, pale brown to brown, 3.3-4.8 (av. 4.1) µm in diam. (Fig. 1O).

***Chaetomium* sp.** Colonies: Surface with dense and orange to yellow aerial mycelia, reverse chocolate brown with yellowish-brown margins (Figs. 1P, Q). Perithecia: Globose to subglobose, dark, with curved hairs covering the surface, 72-94 × 65-75 (av. 81.0 × 70.0) µm (Fig. 1R). Ascospores: Aseptate, lemoniform to oval-shaped, grayish green, 8.4-11.6 × 4.8-6 (av. 10.0 × 5.4) µm (Fig. 1S).

***Colletotrichum chlorophyti* S. Chandra & Tandon** was isolated from sprouts with partial brown spots (Fig. 1T). Colonies: Surface with dense and grayish aerial mycelia, reverse gray (Figs. 1U, V). Acervuli: with brown setae (Fig. 1W). Conidia: Aseptate, falcate, hyaline, guttulate, 19.8-26.5 × 3.6-5.2 (av. 23.7 × 4.5) µm (Fig. 1X).

***Colletotrichum nymphaeae* (Pass.) Aa** was isolated three times from sprouts with partial brown spots (Fig. 1Y). Colonies: surface fluffy and with pale gray aerial mycelia and yellow to orange conidial masses, reverse pale grayish orange (Figs. 1Z-Δ). Acervuli: without seta. Conidia: Aseptate, ellipsoid to oblong or fusiform, hyaline, guttulate, 10.1-16.6 × 3.6-5.6 (av. 12.8 × 4.7) µm (Fig. 1Θ).

***Fusarium equiseti* (Corda) Sacc.** was isolated from sprouts with girdling rot of black matpe (Fig. 2A). Colonies: Surface with sparse and white to pale orange aerial mycelia, reverse pale orange darker than the surface (Figs. 2B, C). Macroconidiogenous cells: Monophialidic, obovoid to obclavate (Fig. 2D). Macroconidia: Falcate, pointed at apices, with basal foot cell, 4-6 septate, hyaline, 36.5-53.6 × 2.8-4.5 (av. 47.5 × 3.6) µm (Fig. 2E). Microconidia: Rare, 1 or 2-septate. Chlamydospores: Chained (Fig. 2F).

***Fusarium oxysporum* Schltdl.** was isolated from sprouts with browning young leaves (Fig. 2G). Colonies: Surface with dense and white aerial mycelia, reverse purple in central area surrounded by a white peripheral area (Figs. 2H, I). Microconidiophores: Short, not blanched (Fig. 2J). Microconidia: Ellipsoid, cylindrical, ovoid, boat-shaped, aseptate, hyaline, 4.5-12 × 2.5-4 (av. 8.5 × 3.5) µm (Figs. 2J, L). Chlamydospores: Apical or intercalary and smooth (Fig. 2K). Macroconidia: Falcate, 3-septate, hyaline, 20.6-47 × 2.8-4 (av. 28.6 × 3.4) µm (Fig. 2L).

***Fusarium* sp.** Conidia: Falcate to boat-shaped, pointed at apices, 3-septate, hyaline, 15-26.2 × 3.1-3.9 (av. 20.0 × 3.6) µm.

***Geotrichum candidum* Link** was isolated three times from sprouts with partial brown spots or grains (Fig. 2M). Colonies: Surface with sparse and white aerial mycelia, reverse white to cream (Figs. 2N, O), with fruit-like aroma. Primary hypae: Dichotomously to trichotomously blanched, hyaline, smooth, 8-10 (av. 8.7) µm wide (Fig. 2P). Conidia: Holoarthric, aseptate, short cylindrical to broadly ellipsoid, smooth, hyaline, 4-11 (-15.2) × 3.4-8 (av. 9.0 × 4.8) µm (Fig. 2Q).

***Macrophomina phaseolina* (Tassi) Goid.** was isolated four times from rotted sprouts dark brown to black in color

Fig. 1. Contamination and spoilage of mung bean sprouts and fungi isolated from them

A: browning roots of sprouts, **B-D**: *Alternaria alternata* (MAFF 239884) isolated from the spoiled sprouts A, **B**: surface side of a colony on a PDA plate, **C**: reverse side of colony B, **D**: conidia, **E**: *Alternaria* sp. (MAFF 243775). **F**: browning of sprouts covered with white mold, **G-J**: *Arthrinium arundinis* (MAFF 238039) isolated from the spoiled sprouts F, **G**: surface side of a colony on a PDA plate, **H**: reverse side of colony G, **I**: conidiophores (p) and young conidia, **J**: mature conidia. **K-M**: *Aspergillus flavus* (MAFF 243495), **K:** conidial heads bearing yellowish-green conidia produced on a PDA plate, **L**: a conidial head bearing phialides (phase contrast optics), **M**: a conidiophore (p) and conidia, **N,O**: *Aspergillus niger* (MAFF 243496), **N**: conidial heads bearing brown conidia produced on a PDA plate, **O**: a conidiophore (p) and conidia. **P-S**: *Chaetomium* sp. (MAFF 243477), **P**: surface side of a colony on a PDA plate, **Q**: reverse side of colony P, **R**: a perithecia covered with hairy hyphae, **S**: ascospores. **T**: browning hypocotyls of sprouts, **U-X**: *Colletotrichum chlorophyti* (MAFF 305748) isolated from the spoiled sprouts T, **U**: surface side of a colony on a PDA plate, **V**: reverse side of colony U, **W**: brown setae on an acervulus, **X**: falcate conidia. **Y**: sprouts with brown spots, **Z-Θ**: *Colletotrichum nymphaeae* (MAFF 306343) isolated from the spoiled sprouts Y, **Z**: colonies developed from the sprouts Y on a PDA plate, **Γ**: surface side of a colony on a PDA plate, **Δ**: reverse side of colony Γ, **Θ**: fusiform conidia. (K, N, R: dissecting microscopy)

or grains (Fig. 2R). Colonies: Grow fast even at 35°C, surface with dense and pale gray aerial mycelia, reverse black (Figs. 2S, T). Pycnidia: Subglobose, black, up to 250 µm in diameter. Microsclerotia: Subglobose to broadly ellipsoid, black, up to 150 µm in diam. (Fig. 2U). Conidia: Aseptate, straight, subcylindrical to oblong or fusiform, hyaline, 13.6-24.1 × 4.7-6.8 (av. 20.0 × 5.6) µm (Fig. 2V).

***Phoma* sp.** Colonies: Surface with sparse and pale gray to white aerial mycelia, reverse white to cream with grayish center (Figs. 2W, X). Pycnidia: Subglobose with a single ostiole framed the outline in dark brown (Fig. 2Y). Conidia: Lemoniform to broadly ellipsoid, hyaline, smooth, 2.7-4.9 × 2-3.7 (av. 3.5 × 2.7) µm (Fig. 2Z).

Phomopsis phaseoli* (Desm.) Sacc. var. *phaseoli (*Diaporthe phaseolorum* (Cooke & Ellis) Sacc. var. *phaseolorum*) Colonies: Surface with dense and pale brown to white aerial mycelia studded with black dots (pycnidia), reverse beige (Figs. 2Γ, Δ). Pycnidia: Gregarious on stromata, black, exuded conidial musses (Fig. 2Θ). Alfa-type conidia: Aseptate, ellipsoid to oblong or fusiform, hyaline, guttulate, 4.9-7 × 2.1-3 (av. 6.0 × 2.6) µm (Fig. 2Λ), without beta-type conidia.

***Phomopsis* sp.** (*Diaporthe* sp.) Colonies: Surface with dense and white aerial mycelia, reverse yellowish-brown with dark brown sectoring (Figs. 3A, B). Alfa-type conidia: Aseptate, ellipsoid to oblong or fusiform, hyaline, guttlate, 6-7.7 × 2-3.1 (av. 7.0 × 2.5) µm (Fig. 3C), without beta-type conidia.

***Trichoderma* sp.** Colonies: Surface with dense grayish green aerial mycelia around, reverse similar to the surface (Figs. 3D, E). Conidiophores: Cylindrical, tapered to distal end, hyaline, smooth, 3-3.9 µm wide, 54-74 µm long. Phialides: Trichotomously arranged, obclavate, hyaline, smooth, 6.5-14.8 × 2.6-3.3 (av. 10.0 × 3.0) µm (Fig. 3F). Conidia: Lemoniform to ellipsoid, hyaline, smooth, 4.5-6.3 × 2.9-4.1 (av. 5.4 × 3.5) µm (Fig. 3G).

***Rhizoctonia solani* J.G. Kühn (AG-4, HG-I)** was isolated from partially browning sprouts (Fig. 3H) or grains: Colonies: Surface pale brown to brown, with entire, secreted brown pigment dispersively into the medium (Fig. 3I). Hyphae: Multinuclear, blanched at right angles to primary hyphae, constricted at blanch base, with septa near blanches, 4.4-10.3 (av. 7.7) µm in diam. (Fig. 3J).

***Rhizoctonia solani* J.G. Kühn (AG-4, HG-III)** Colonies: Surface with sparse aerial mycelia, pale brown, slightly frosty, forming brown microscleroria covered with downy hyphae in a radial pattern (Fig. 3K). Hyphae: Similar to those of HG-I, 5.4-10.3 (av. 7.7) µm in diam. (Fig. 3L).

***Globisporangium ultimum* var. *ultimum* (Trow) Uzuhashi, Tojo & Kakish.** (*Pythium ultimum* Trow var. *ultimum*) Colonies: Surface with sparse and white mycelia, reverse also entirely white (Figs. 3M, N). Hyphal swellings: Intercalary or terminal, broadly ellipsoid to lemoniform, hyaline, smooth, (17.5-) 19.1-24.1 (-26.4) (av. 20.7) µm in diam. Oogonia: Terminal sometimes intercalary globose, smooth, 20.2-23.6 (av. 21.9) µm in diam. Antheridia: 1-3 per oogonium, sac-like, often monoclinous or diclinous, 10.3-12.1 × 5.5-8.7 (av. 10.9 × 7.6) µm (Fig. 3O).

***Lichtheimia ramosa* (Zopf) Vuill.** (*Absidia corymbifera* var. *ramosa* (Zopf) Coudert) was isolated from entangled and rotted sprouts (Fig. 3P). Colonies grew fast even at 37°C, surface with sparse but tall and pale brown mycelia, reverse grayish beige (Figs. 3Q, R). Rhizoids: Simple, with short blanches. Sporangiophores: Often curved and blanched, smooth, hyaline 6-18 µm wide, up to 165 µm long. Sporangia: Globose to broadly ovoid, grayish brown, 38-74 (-104) (av. 55) µm in diam. Columella: Broadly ellipsoid to ovate, hyaline 30-54 (-64) (av. 42) µm in diam. Sporangiospores: Broadly ellipsoid to short cylindrical, hyaline to pale gray, smooth, 3.5-4.5 × 2.8-4 (av. 4.1 × 3.2) µm (Figs. 3S, T).

***Rhizopus oryzae* Went & Prins. Geerl.** was isolated from sprouts entangled with black mold (Fig. 3U). Colonies grew fast even at 40°C, surface with sparse but tall and dark gray mycelia, reverse cream (Figs. 3V, W). Rhizoids: Simple, with several short blanches. Sporangiophores: Cylindrical, smooth, hyaline, 12-20 (av. 15.2) µm wide, 1.5 mm long (Fig. 3X). Sporangia: Globose, grayish black, 100-160 (av. 125.0) µm in diameter (Figs. 3X, Y) Columella: Ellipsoid with attenuated base, pale grayish brown, (52-) 68-140 (av. 92.0) µm in height. Sporangiospores: Subglobose to broadly ellipsoid or angular spherical, pale brown, striate, 4.5-10.5 (-15.6) × 4-9 (av. 8.0 × 6.2) µm (Fig. 3Z).

3. Fungi isolated from soybean sprouts

At least 15 fungal isolates identified as seven species of six genera were obtained from soybean (*G. max*). The morphology of the species was described as follows, and the results of the BLASTn search are summarized in Table 2.

***Cercospora kikuchii* (Tak. Matsumoto & Tomoy.) M.W. Gardner** was isolated from grains with purple stain (Fig. 4A). Colonies grew slowly even at optimal temperatures, surface with dense and white mycelia, reverse reddish-brown (Figs. 4B, C). Conidia: Filiform, straight to slightly curved, with truncate base, multi-septate, hyaline, smooth, (18-) 89-118 × 2-3 (av. 97 × 2.1) µm (Fig. 4D).

***Diaporthe phaseolorum* var. *caulivora* Athow & Caldwell** was observed after incubation of contaminated grains on agar plates (Fig. 4E). Colonies: Surface with dense but margin sparse and white to pale beige mycelia, reverse pinkish beige (Figs. 4F, G). Perithecia: Flask-shaped, 194-299 × 184-278 (av. 230.0 × 211.0) µm with long beak (270-560 µm) (Fig. 4H). Asci: Clavate with apical ring and eight spores, 24.7-42.6 × 4.1-8.1 (av. 31.7 ×

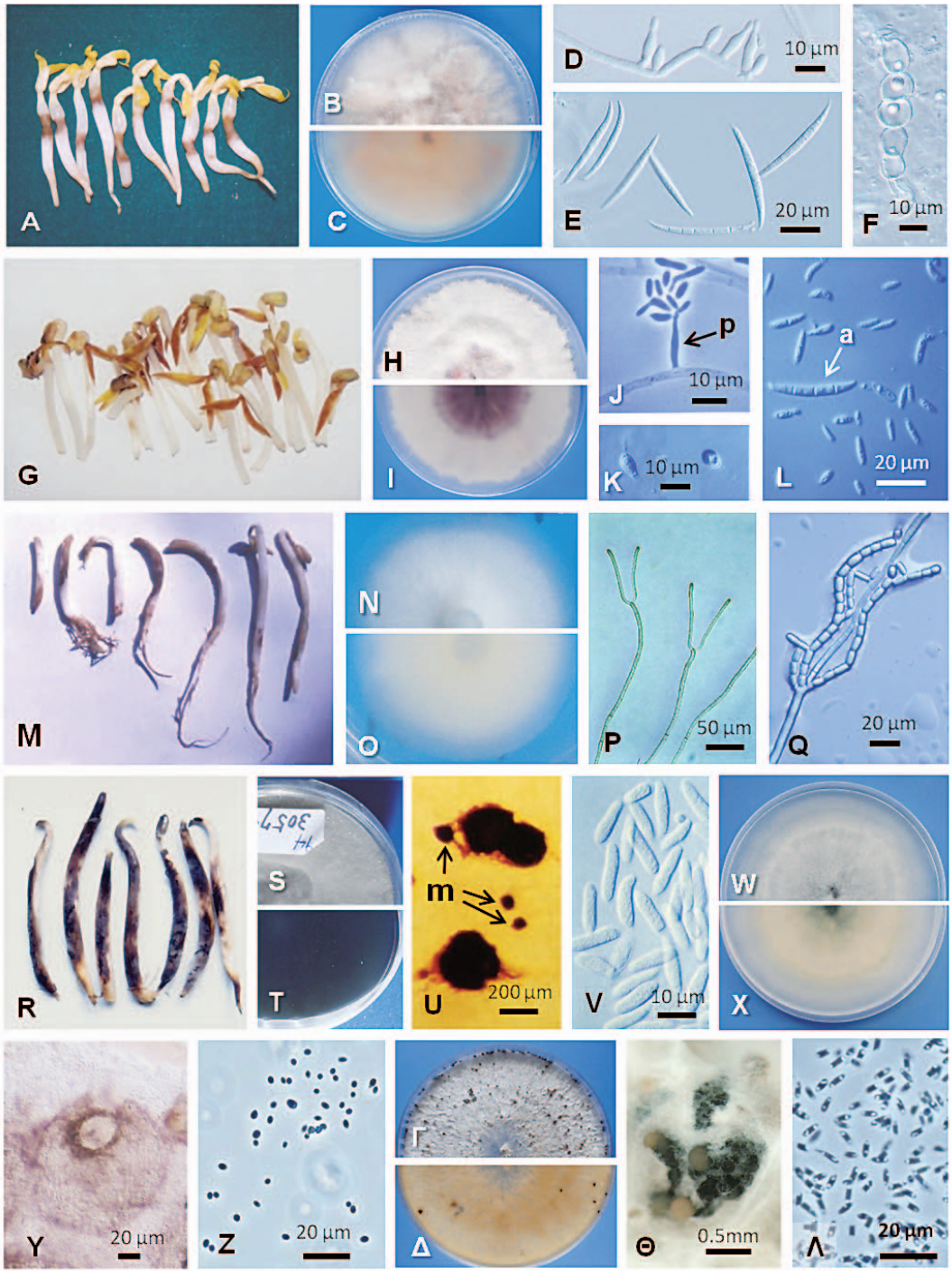

Fig. 2. Contamination and spoilage of mung bean or black matpe bean sprouts and fungi isolated from them

A: brown girdling hypocotyls of sprouts (black matpe), **B-F**: *Fusarium equiseti* (MAFF239547) isolated from the spoiled sprouts A, **B**: surface side of a colony on a PDA plate, **C**: reverse side of colony B, **D**: monophialides, **E**: macroconidia, **F**: chlamydospores. **G**: browning of young leaves from sprouts, **H-L**: *Fusarium oxysporum* (MAFF 239895) isolated from the spoiled sprouts G, **H**: surface side of a colony on a PDA plate, **I**: reverse side of colony H, **J**: a microconidiophore (p) and microconidia, **K**: chlamydospores, **L**: macroconidia (a) and microconidia. **M**: browning hypocotyls of sprouts, **N-Q**: *Geotrichum candidum* (MAFF 239885) isolated from the spoiled sprouts M, **N**: surface side of a colony on a PDA plate, **O**: reverse side of colony N, **P**: dichotomous blanch of hyphae, **Q**: conidia cut from hyphae. **R**: blackened hypocotyls of sprouts, **S-V**: *Macrophomina phaseolina* (MAFF 305746) isolated from the spoiled sprouts R, **S**: surface side of a colony on a PDA plate, **T**: reverse side of colony S, **U**: pycnidia and microsclerotia (m), **V**: conidia. **W-Z**: *Phoma* sp. (MAFF 243774), W: surface side of a colony on a PDA plate, **X**: reverse side of colony W, **Y**: pycnidium, **Z**: conidia. **Γ-Λ**: *Phomopsis phaseoli* var. *phaseoli* (MAFF 242916), **Γ**: surface side of a colony on a PDA plate, **Δ**: reverse side of colony Γ, **Θ**: conidial masses exuded from black pycnidia, **Λ**: conidia. (J, Z, Λ: phase contrast optics)

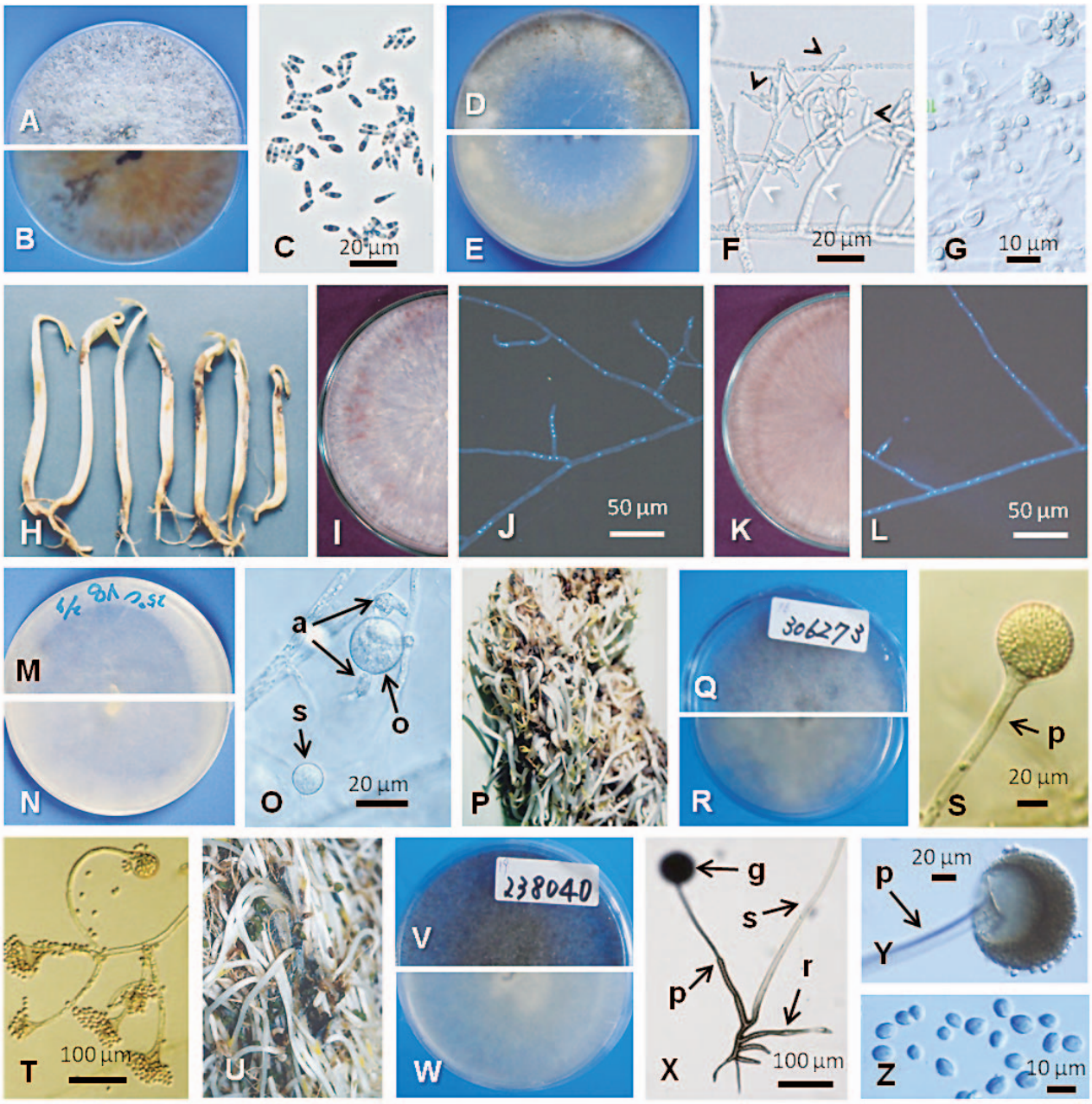

Fig. 3. Contamination and spoilage of mung bean sprouts and fungi isolated from them

A-C: *Phomopsis* sp. (MAFF 243678), **A**: surface side of a colony on a PDA plate, **B**: reverse side of colony A, **C**: Conidia, **D-G**: *Trichoderma* sp. (MAFF 242918), **D**: surface side of a colony on a PDA plate, **E**: reverse side of colony D, **F**: conidiophores (white arrowheads) and phialides (black arrowheads), **G**: conidia, **H**: partially brown hypocotyl of sprouts, **I**, **J**: *Rhizoctonia solani* AG-4, HG-I (MAFF 239817), **I**: surface side of a colony on a PDA plate, **J**: hyphal nuclei (sky blue dots) stained with DAPI, **K**, **L**: *Rhizoctonia solani* AG-4, HG-III (MAFF 241477), **K**: surface side of a colony on a PDA plate, **L**: hyphal nuclei (sky blue dots) stained with DAPI. **M-O**: *Globisporangium ultimum* var. *ultimum* (MAFF 241478), **M**: surface side of a colony on a PDA plate, **N**: reverse side of colony M, **O**: an oogonium (o) with antheridia (a) and hyphal swellings (s). **P**: entangled and rotted sprouts, **Q-T**: *Lichtheimia ramosa* (MAFF 306273) isolated from the spoiled sprouts P, **Q**: surface side of a colony on a PDA plate, **R**: reverse side of colony Q, **S**: a sporangiophore (p) and a sporangium, **T**: blanched and curved sporangiophore and sporangiospores. **U**: rotted sprouts entangled with black mold, **V-Z**: *Rhizopus oryzae* (MAFF 238040) isolated from the spoiled sprouts U, **V**: surface side of a colony on a PDA plate, **W**: reverse side of colony V, **X**: stolon (s), rhizoid (r), sporangiophore (p) and sporangium (g), **Y**: a sporangiophore (p) and a sporangium, **Z**: sporangiospores**.** (C: phase contrast optics, J, L: fluorescence microscopy).

5.8) μm (Fig. 4I). Ascospores: Oblong to fusiform, median septate, hyaline, with a few guttules, 6.6-10.4 × 3-4.5 (av. 8.2 × 3.5) μm (Fig. 4I).

***Fusarium graminearum* Schwabe** (*Gibberella zeae* (Schwein.) Petch) was isolated from grains with a reddish-brown stain when they were incubated under moist conditions (Fig. 4J). Colonies: Surface with dense and white to vinaceous red mycelia, reverse pinkish-red (Figs. 4K, L). Macroconidogenous cells: Monophialidic, ellipsoid, verticillate on conidiophores (Fig. 4M). Macroconidia: Falcate, pointed at apices, with basal foot cells, (3-) 5-7 septate, hyaline, 40.1-70 × 4.5-6.3 (av. 52.4 × 5.2) μm (Fig. 4N). Chlamydospores: Subglobose to broadly ellipsoid, chained a few cells, hyaline, smooth, 7-13.6 × 6-8.6 (av. 9.0 × 8.6) μm. Perithecia: Subglobose, reddish-brown, containing many clavate asci; each containing eight 3-4-celled ascospores (Figs. 4O-R).

***Fusarium oxysporum* Schltdl.** Morphological characteristics were described earlier. Microconidia: 5.8-11.5 × 2.6-4 (8.7 × 3.4) μm. Macroconidia: 23.8-42.8 × 3-4.5 (av. 26.8 × 3.9) μm. Chlamydospores: Present.

***Penicillium oxalicum* Currie & Thom** was isolated from rotted sprouts yellowish-brown in color and water-soaked (Fig. 4S). Colonies: Surface powdery, deep green in maturing (Fig. 4T). Conidiophores: Long cylindrical, hyaline, smooth, 3.8-4.4 μm wide, more than 100 μm long. Penicilli: Bi-verticillate. Phialides: Cylindrical to clavate, hyaline, smooth, 7.2-11.9 × 2.8-3.8 (av. 9.6 × 3.4) μm. Conidia: Lemoniform to ellipsoid, hyaline, smooth, 4.5-6.3 × 2.9-4.1 (av. 5.4 × 3.5) μm (Fig. 4U).

***Phoma medicaginis* Malbr. & Roum.** was isolated from grains with brown spots or browning husks with many pycnidia (Figs. 4V, W). Colonies: Surface with sparse and pale brown mycelia in the central area surrounded by a beige marginal area (Fig. 4X). Pycnidia on PDA: Globose to subglobose or ellipsoid with one or a few ostiole(s), 83-233 × 70-210 (av. 170.0 × 125.0) μm, produced pinkish conidial masses (Figs. 4Y, Z). Conidia: Formed from phialides, aseptate, ellipsoid to cylindrical, hyaline, 3.4-10.5 × 1.7-4.4 (av. 5.8 × 2.8) μm (Fig. 4Γ).

***Phomopsis phaseoli* var. *sojae* (Lehman) Sacc.** (*Diaporthe phaseolorum* var. *sojae* (Lehman) Wehm.) Colonies: Surface with dense and white mycelia, reverse pale beige (Figs. 4Δ, Θ). Conidia: Aseptate, ellipsoid to oblong or fusiform hyaline, guttulate, 5.8-8.3 × 1.9-3.1 (av. 7.0 × 2.4) μm (Fig. 4Λ).

***Syncephalastrum racemosum* Cohn ex J. Schröt.** Sporangiophores: With short branches bearing apical vesicles. Vesicles: Globose, 41-65 μm in diameter. Merosporangia: Cylindrical, containing up to ten spores, 14-25 μm long. Merospores: Globose or ovoid, smooth, hyaline, 2.8-4.3 × 2.5-3.4 (av. 3.5 × 2.8) μm (Fig. 4Σ).

Discussion

Fifteen and five fungal species were first isolated from *Vigna* spp. and soybean sprouts including their ingredient grains, respectively. Neither *Fusarium solani* nor *Colletotrichum gloeopsporioides,* which were reported from *Vigna* bean sprouts previously (Aoki et al., 1986, 2000; Furuya et al., 2002), was isolated in this study. *Colletotrichum* sp. strain MAFF 305748 (Aoki et al., 1986) was re-identified as *Colletotrichum chlorophyti* S. Chandra & Tandon based on its TUB2 sequence and morphology. *Fusarium equiseti* and *Phomopsis phaseoli* var. *phaseoli* were unique to *Vigna* spp. ingredients from Thailand and Myanmar, respectively. No isolates of *Fusarium moniliforme*, *F. solani* and *Pythium deliense*, which have been reported from soybeans (Oh and Park, 1996, Yun and Kim, 2003), were obtained in this study. *Diaporthe phaseolorum* var. *caulivora* was only isolated from soybeans introduced from the United States. In contrast, *Fusarium graminearum* was found in soybean grains from China and the United States, suggesting the universality of the fungus, although no disease of soybean caused by *F. graminearum* has been reported in Japan (Anonymous 2012).

More than 80% of the species were identified as ascomycetous fungi, although few produced teleomorphs. Three zygomycetous fungi, *Lichtheimia ramose*, *Rhizopus oryzae* and *Syncephalastrum racemosum*, which entangled bean sprouts characteristically with dark mycelia (Figs. 3P, 3U), are all fast colonizers under moist and warm conditions. One Oomycete, *Globisporangium ultimum* var. *ultimum* (*Pythium ultimum* var. *ultimum*), was newly identified from the mung bean. The species might originate from the water used to process bean sprouts, because *Globisporangium* spp. had not been recognized as seed-borne but rather soil-borne (Plaats-Niterink, 1981) and the genus was recently separated from *Pythium*, which is adapted to aquatic environments (Plaats-Niterink, 1981; Uzuhashi et al., 2010).

Aspergillus flavus (MAFF 239890, MAFF 239891), *Aspergillus terreus* Thom (MAFF 239893) and *Paecilomyces lilacinus* (Thom) Samson (MAFF 239892) were isolated from waste water from a bean sprout plant (T. Yaguchi, unpublished data). Therefore, most fungi isolated from bean sprouts and ingredient grains except for *A. flavus*, seemed to originate from the grains. The plant pathogen *Penicillium oxalicum* was first isolated from soybean sprouts and its principal habit appeared to be decaying vegetation (Pitt, 1979). Approximately 70% of fungi isolated from *Vigna* spp. and soybean are recognized as plant pathogens in Japan (Anonymous 2012) or abroad (Damm et al., 2009; Kulik, 1989; Hartman et al., 1999, Tables 1, 2). In addition, at least 14 species are known to be seed-borne (Malone et al., 1997; Hartman et al., 1999; Tables 1, 2). It is remarkable from a plant quarantine perspective that the

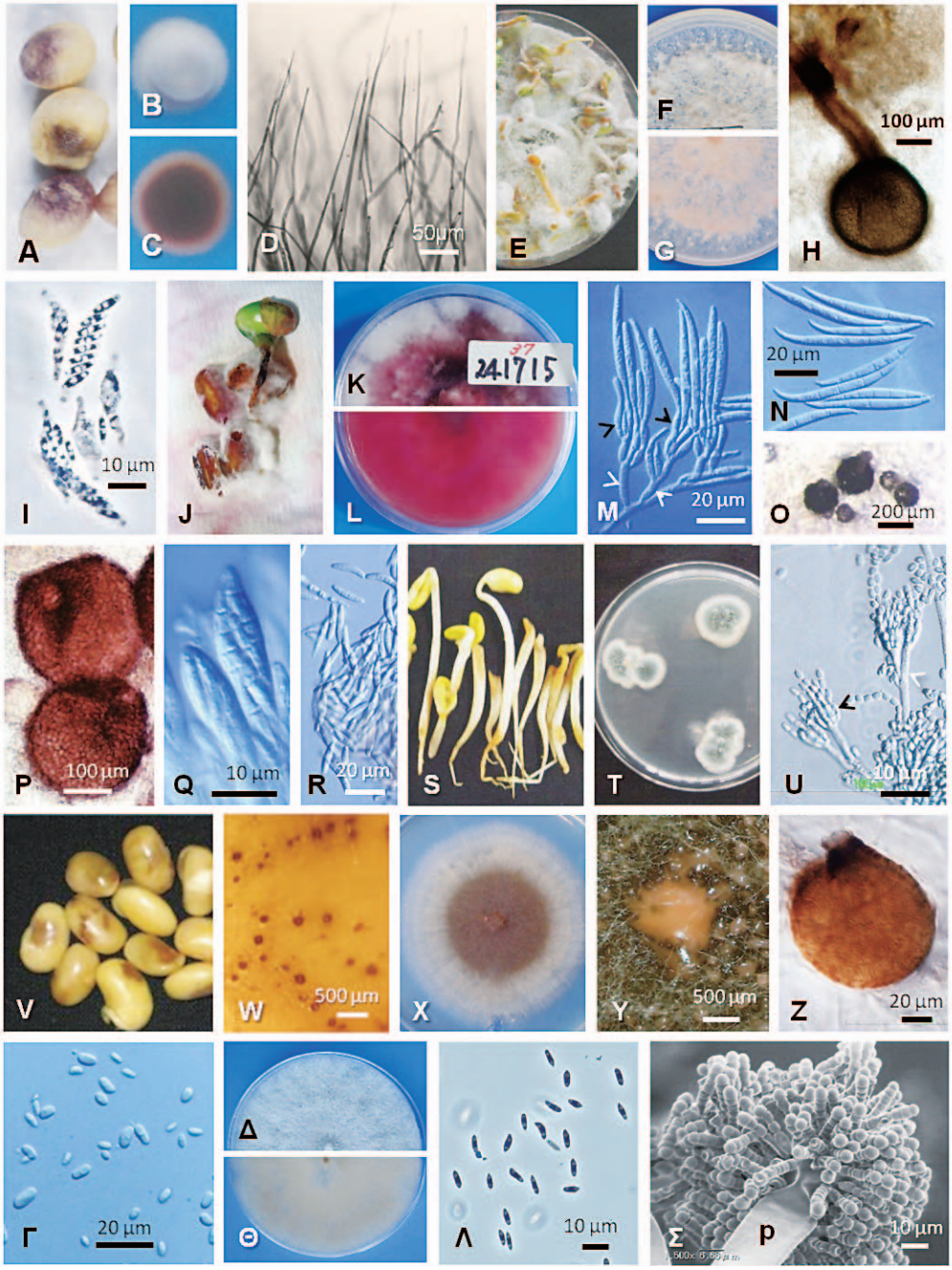

Fig. 4. Contamination and spoilage of soybean sprouts and fungi isolated from them

A: grains with purple to dark brown lesions, **B-D**: *Cercospora kikuchii* (MAFF 239883) isolated from the spoiled grains A, **B**: surface side of a colony on a PDA plate, **C**: reverse side of colony B, **D**: needle-like conidia produced on PDA (courtesy of Dr. Y. Fujita). **E**: mold developed from contaminated soybean grain on a PDA plate, **F-I**: *Diaporthe phaseolorum* var. *caulivora*, **F**: surface side of a colony on a PDA plate, **G**: reverse side of colony F, **H**: a perithecium produced in PDA, **I**: asci, **J**: rotted sprouts in reddish-brown color, **K**, **L**: *Fusarium graminearum* (MAFF 241715) isolated from the spoiled grains J, **K**: surface side of a colony on a PDA plate, **L**: reverse side of colony K. **M-R**: *F. graminearum* (*Gibberella zeae*) (MAFF 238042), **M**: conidiophores (white arrowheads), phialides (black arrowheads) and young macroconidia, **N**: mature macroconidia, **O**: perithecia produced on a SNA plate with filter paper, **P:** mature perithecia, **Q**: asci, **R**: ascospores. **S**: water-soaked rot of hypocotyls, **T**, **U**: *Penicillium oxalicum* (MAFF 242579) isolated from the spoiled sprouts S, **T**: mold developed from the spoiled sprouts S, **U**: conidiophores (white arrowhead), phialides (black arrowhead) and conidia. **V**: brown spots on grain husks, **W**: pycnidia produced on the spot V, **X-Γ**: *Phoma medicaginis* (MAFF 240348) isolated from the pycnidia W, **X**: surface side of a colony on a PDA plate, **Y**: conidial masses, **Z**: a pycnidium, **Γ**: conidia. **Δ-Λ**: *Phomopsis phaseoli* var. *sojae* (MAFF 243679), **Δ**: surface side of a colony on a PDA plate, **Θ**: reverse side of colony Δ, **Λ**: conidia. **Σ**: a scanning electron micrograph of sporangiophore (p) and many merosporangia of *Syncephalastrum racemosum* (MAFF 241792). (I, Λ: phase contrast optics, O, W, Y: dissecting microscopy)

ingredients of bean sprouts are all imported from other countries into Japan. For example, *Diaporthe phaseolorum* var. *caulivora*, which was isolated from the soybean in this study, is an important pathogen in North and South America (Kulik, 1989; Hartman et al., 1999; Costamilan et al., 2008), but has never been found in Japan. Such infested grains imply risks; not only in bean sprout production but also in crop protection of the importing countries. Inoculation experiments with representative strains of each species are needed to estimate the risks, although spoilage and rot reproduced by inoculations of the mung bean and soybean sprouts with some strains were preliminarily reported (Sato et al., 2008a, b). The ingredient grains should be imported after complete sterilization in the producing countries, because they could carry exotic plant pathogens.

Several species of the isolated fungi are known to produce mycotoxins (Samson & Reene-Hoekstra, 1988). Two strains of *Fusarium graminearum*, MAFF 241713 and MAFF 238042, isolated from the soybean were reported to produce very high concentrations of deoxynivalenol, 45.4 and 21.6 ppm, respectively (Saito, 2009). Because grains carrying *F. graminearum* became reddish during incubation (Fig. 4J), those with such an appearance should be immediately eliminated from processing. *Aspergillus flavus*, which was found in mung bean sprouts, is a well-known aflatoxin producer (Samson & Reene-Hoekstra, 1988). The fungus, as mentioned above, was also found in a bean sprouts plant. There is therefore a need not only to sterilize the grains, but also to thoroughly clean the processing plant facilities.

As mentioned earlier, sterilization techniques, such as soaking in bleaching fluid, exposure to ammonia gas or allylisothiocyanate and microwave heating together with steam, were attempted to eliminate the spoiling fungi from the ingredient grains in Japan (Aoki et al., 1986, 2000; Furuya et al., 2002, 2003). The sterilization effects of the various techniques need to be re-examined with the strains of fungi isolated in this study to enhance their practicality.

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