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INTERNATIONAL STANDARD FOR PHYTOSANITARY MEASURES 27

DIAGNOSTIC PROTOCOLS

DP 19:
Sorghum halepense

Produced by the Secretariat of the
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ISPM 27
Diagnostic protocols for regulated pests

DP 19: *Sorghum halepense*

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1. Pest Information

Sorghum halepense (Johnsongrass) is a perennial grass with a ribbed leaf sheath, conspicuous midrib, large, purplish panicles, and far-reaching rhizomes (Figures 1 and 2). Its origin remains unclear, but some authors suggest that it originated from the hybridization of *Sorghum arundinaceum* and *Sorghum propinquum* through chromosome doubling (chromosomes: 2n = 4x = 40) (Ng'uni *et al.*, 2010). *S. halepense* is native to the Mediterranean area (Meredith, 1955) and has been introduced to other regions (Bor, 1960). It has become widespread, and is distributed from latitude 55° north to 45° south. It is best adapted to warm, humid areas with summer rainfall, areas with a high water table, and irrigated fields in subtropical zones. *S. halepense* is one of the most malignant weeds worldwide, impacting more than 30 cereal, vegetable and fruit crops (Holm *et al.*, 1977). It also threatens biodiversity in at least 50 countries in temperate and tropical areas throughout the world, including countries in which it is a native species (Holm *et al.*, 1977).

The main factors affecting the pest risk of *S. halepense* as a pest of plants are that: (1) it has a high asexual and sexual reproductive capacity; (2) its seeds can be dormant and are long-lived, and can move with traded commodities (Warwick and Black, 1983); (3) it has strong competitive ability and causes great yield loss in crops (Follak and Essl, 2012); (4) it is an alternate host of numerous pathogen species; (5) it has allelopathic effects and is toxic to livestock (da Nobrega *et al*., 2006); (6) it has developed resistance to a wide range of herbicide groups (Heap, n.d.); and (7) it has self-compatibility but readily crosses with related species, which may result in more invasive hybrids or cause gene introgression of crop species (Warwick and Black,1983; Arriola and Ellstrand, 1996).

S. halepense is able to reproduce by rhizomes or seeds. Rhizomes readily sprout and can be distributed by tillage. An individual *S. halepense* plant is able to produce as many as 28 000 seeds in a growing season. These seeds are able to survive and germinate under most environmental conditions. The seeds are caryopses and are brown, obovate, 3 × 1.6 mm in size, with an elliptic sessile spikelet that is appressed pubescent (Table 1).

Seeds are the main means of spread of *S. halepense*, and they are readily distributed naturally by wind and water as well as by birds and other animals. More importantly, the seeds are frequently disseminated by human activity as a contaminant of commodities traded around the world; in particular, crop seeds and raw grains, such as *Sorghum bicolor* (sorghum), *Glycine max* (soybean), *Zea mays* (maize), *Triticum aestivum* (wheat) and *Sesamum indicum* (sesame), as well as forage, *Gossypium* spp. (cotton) and birdseed mixes.

2. Taxonomic Information

Name: *Sorghum halepense* (L.) Pers., 1805

Synonyms: *Holcus halepensis* L., 1753

Taxonomic position: Plantae, Angiospermae, Monocotyledonae, Poales, Poaceae

Common names: Johnson grass, Johnsongrass (English)

3. Detection

Common survey methods for herbaceous species may be adopted for the detection of *S. halepense* in the field. In order to detect seeds of *S. halepense* in crop seeds, an inspection procedure should be followed in which a composite sample is prepared for laboratory analysis and sieve detection (ISTA, 2014).

3.1 Preparation of samples for laboratory analysis

General guidance on sampling methodologies is described in ISPM 31 (*Methodologies for sampling of consignments*). The sample for examination should be approximately 1 kg. Remaining sample material

should be labelled and conserved in paper bags or glassware free from moisture for possible further checking.

3.2 Sieve detection

A set of three sieves should be assembled with decreasing aperture sizes according to the seeds or grains being sampled, within an overall range of 2 mm to 10 mm. The largest aperture sieve is placed on top of the second largest sieve, with the smallest sieve on the bottom. The sample for examination is placed in the top sieve and the sieve set assembly is covered before sieving the sample through it. After sieving, the material remaining in each sieve layer is collected and placed onto white plates for visual examination. The suspected *S. halepense* seed fragments and seeds (resembling those shown in Figure 3) are selected for further identification.

4. Identification

Identification of *S. halepense* seeds is the main task and is commonly based on morphology. For suspected seeds with intact glumes and upper lemmas, morphological identification methods (section 4.1) are reliable. However, the fruits and seeds collected may be incomplete and parts of their characters unclear. In such cases, molecular (section 4.2) or biochemical (section 4.3) identification methods may need to be used. Seeds may also be sown and grown into seedlings and mature plants, either of which can be morphologically (section 4.4) or cytologically (section 4.5) examined for taxonomic traits and subsequently identified as a complement. Figure 4 presents a flow chart for the identification of *S. halepense*.

S. halepense is prone to be confused with five related species in the genus *Sorghum*:

- *S.* × *almum* Parodi (*S. bicolor* subsp. *drummondii* (Nees ex Steud.) de Wet ex Davidse), 1943
- *S. bicolor* (L.) Moench, 1794
- *S. propinquum* (Kunth) Hitchcock, 1929
- *Sorghum* spp. hybrid cv. Silk (silk sorghum), a hybrid between Krish hybrid sorghum (*S. halepense* × *S. roxburghii*) and *S. arundinaceum*, 1978 (CSIRO, 1978; Flora of China Editorial Committee, 1997, 2013; Ross, 1999; Barkworth, 2013).
- *S. sudanense* (Piper) Stapf, 1917.

This diagnostic protocol compares *S. halepense* with the above five closely related species. Detailed descriptions of plant morphological characteristics can be found for *S. halepense* in Holm *et al.* (1977) and Flora of China Editorial Committee (1997, 2013); for *S.* × *almum*, *S. bicolor*, *S. propinquum* and *S. sudanense* in Flora of China Editorial Committee (1997, 2013); and for *Sorghum* spp. hybrid cv. Silk in CSIRO (1978) and Ross (1999).

4.1 Morphological identification of seeds

The caryopsis of *S. halepense* is brown, obovate, 2.6–3.2 mm in length and 1.5–1.8 mm in width; obtuse in the apex with persistent style; hilum rotund, deep purple–brown; ventral side flat; embryo oval or obovate, with length approximately one-third to half of the caryopsis (Figures 2 and 3).

S. halepense seeds can be identified based on characteristics of the glume and upper lemma (Tables 1 and 2). A key for species identification can be used to distinguish similar species if a seed is not easily matched to the description of characteristics in Tables 1 and 2.

Table 1. Comparison of the sessile spikelet, caryopsis and seed weight in *Sorghum halepense* and five related species

Species	Sessile spikelet	Caryopsis	Weight of 1 000 seeds (g, approximate)
S. halepense	Elliptic or ovate, (3.8) 4–5 (6.5) mm in length, appressed pubescent	Dark brown, obovate or elliptic, 2.6–3.2 mm in length and 1.5–1.8 mm in width	4.9
S. × *almum*	Elliptic to oblong, 4.5–6.5 mm in length, short pubescent	Red–brown, broadly ovate or oval, 3.3–4 mm in length and 2–2.3 mm in width	6.6
S. propinquum	Ovate, or broadly ovate, 3.8–5 mm in length, bearded	Brown, broadly ovate or broadly oval, approximately 2 mm in length and 1.5 mm in width	3.8
S. sudanense	Elliptic, (5) 6–8 mm in length, sparsely pubescent	Red–brown, broadly ovate, 3.5–4.5 mm in length, 2.5–2.8 mm in width	10–15
S. bicolor	Elliptic to oblong or ovate, (3) 4.5– 6 (10) mm in length, densely hispid, or pubescent to glabrous	Pink to red–brown, ovate, 3.5–4 mm in length, 2.5–3 mm in width	>20
Sorghum spp. hybrid cv. Silk	Oval, approximately 3.8 mm in length, short pubescent	Yellow or yellow–brown, broadly ovate, 2.5–4 mm in length and 1.7–2.5 mm in width	4.2

Source: Based on Holm *et al.* (1977), Sun *et al.* (2002), Qiang (2009), Barkworth (2013), Flora of China Editorial Committee (2013) and Clayton et al (2016).

Table 2. Comparison of the glume and upper lemma of seeds in *Sorghum halepense* and five related species

	Glume	Lower glume	Upper glume	Upper lemma
S. halepense	Subleathery, tawny, red–brown or purple–black	Apex clearly tridenticulate, 5–7-veined, dorsum ciliary but the rest glabrous	3-veined	Triangular lanceolate, apex bilobed and awned or not; awn 10–16 mm
S. × *almum*	Chartaceous or subleathery, dark brown	Apex slightly tridenticulate, 5–7-veined, dorsum ciliary but the rest glabrous	3-veined	Lanceolate, apex obtuse or slightly acute, bilobed, awned; awn approximately 15 mm
S. propinquum	Subleathery, dark brown with inconspicuous crossveins	9–11-veined, apex acute to apiculate or tridenticulate, pubescent	7-veined	Lanceolate, approximately 3.5 mm in length, acute or emarginate, awnless

	Glume	Lower glume	Upper glume	Upper lemma
S. sudanense	Leathery, lemon yellow to red–brown	Apex bidenticulate, 11–13-veined, usually with crossveins, dorsum short ciliary	5–7-veined, with crossveins	Ovate or elliptic, apex bilobed, awned; awn 10–16 mm
S. bicolor	Leathery, pink to red–brown	Apex acute or tridenticulate, 12–16-veined with crossveins, dorsum dense ciliary	7–9-veined	Lanceolate to long oval, 2–4-veined, apex bilobed, awned; awn approximately 1 mm
Sorghum spp. hybrid cv. Silk	Leathery, tawny, red–brown or purple–black	Apex slightly tridenticulate, 5–7-veined, dorsum ciliary but the rest pubescent	3-veined	Broad lanceolate, apex slightly bilobed, awnless

Source: Based on Holm *et al.* (1977), Sun *et al.* (2002), Qiang (2009), Barkworth (2013) and Flora of China Editorial Committee (2013).

4.1.1 Key to the seed morphology of *Sorghum halepense* and five related species

Based on Holm *et al.* (1977), Qiang (2009) and Flora of China Editorial Committee (2013).

1. Glume with clear crossveins; lower glume with more than 11 veins; large seed weight (1 000-seed weight >10 g) **2**

– Glume with no clear crossveins; lower glume with 11 or fewer veins; small seed weight (1 000-seed weight <8 g) **3**

2. Lower glume 11–13-veined, with veins extending to the base; upper glume 5–7-veined, with clear ridge..... ***S. sudanense***

– Lower glume 12–16-veined, with veins not clear on the lower part; upper glume 7–9-veined, with inconspicuous ridge near the top ..... ***S. bicolor***

3. Lower glume 9–11-veined..... ***S. propinquum***

– Lower glume 5–7-veined..... **4**

4. Glume chartaceous or subleathery; upper lemma lanceolate, persistent rachilla rough in the fracture ..... ***S.* × *almum***

– Glume leathery; upper lemma broad lanceolate or triangular lanceolate, persistent rachilla neat in the fracture **5**

5. Lower glume with blurry tridenticulate apex; upper lemma broadly lanceolate..... ..... ***Sorghum* spp. hybrid cv. Silk**

– Lower glume with distinct tridenticulate apex; upper lemma triangular lanceolate ..... ***S. halepense***

4.2 Molecular identification of seeds

Two molecular tests have been referred to support or verify morphological identification of seeds of *S. halepense* in the case of uncertainty of visible morphological characters or for identifying partial seeds. For these methods, at least 0.05 g seeds is needed.

In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

4.2.1 Methods based on DNA markers

For DNA extraction from seed samples, refer to the source paper of the molecular method for the specific technique used (Chen *et al.*, 2009). The method described by Moller *et al.* (1992) is recommended for DNA microextraction from seeds of *Sorghum* species[1]. If more than one seed is included in the extraction, the DNA may comprise a mixture of species. Under certain circumstances, DNA may be extracted from seedlings grown from seed samples.

4.2.1.1 ISSR markers

The method of Fang *et al.* (2008) is based on inter-simple sequence repeat (ISSR) markers. It was evaluated for discriminating the following *Sorghum* species (the origin of the samples used are given in parentheses): *S. saccharatum* (China); *Sorghum* hybrid *S. sudanense* × *S. bicolor*, *S. sudanense* or *S. halepense* (United States of America); *S. bicolor* (Afghanistan); and *S.* × *almum* (Australia). At least ten seeds are needed for each sample.

The ISSR method consists of two separate amplification procedures, each with a single polymerase chain reaction (PCR) primer. The primers are as described by Fang *et al.* (2008):

IR89: 5′-VBVATATATATATATAT-3′

IS16: 5′-AGAGAGAGAGAGACC-3′

Reactions are carried out in a reaction mixture made up to a volume of 20 µl with double-distilled (dd)H_2O and containing: 1× PCR buffer, 2.0 mM $MgCl_2$, 250 µM dNTPs, 400 nM primer, 30 ng DNA template and 1.5 U Taq DNA polymerase. The cycling parameters are 12 min at 94 °C, followed by 40 cycles of (30 s at 94 °C, 30 s at 48 °C and 1 min at 72 °C) and a final step of 12 min at 72 °C. The PCR products are analysed by gel electrophoresis.

The IR89 primer produces 1 500 base pair (bp) and 100 bp amplicons, and the IS16 primer produces 1 200 bp, 1 100 bp, 850 bp and 400 bp amplicons. The *Sorghum* species considered in this diagnostic protocol have the following band patterns:

- *S. halepense:* a single band, 1 500 bp
- *S.* × *almum:* two bands, 1 500 bp and 400 bp
- *S. bicolor:* four bands, 1 200 bp, 1 100 bp, 400 bp and 100 bp
- *Sorghum* hybrid (*S. bicolor* × *S. sudanense*): five bands, 1 200 bp, 1 100 bp, 400 bp, 850 bp and 100 bp
- *S. saccharatum:* three bands, 1 200 bp, 400 bp and 100 bp
- *S. sudanense:* two bands, 400 bp and 100 bp.

4.2.1.2 SCAR markers

The method of Zhang *et al.* (2013) is based on sequence characterized amplified region (SCAR) markers. It was evaluated for discriminating *S. halepense* from 11 other *Sorghum* species, as follows (the origin of the samples used are given in parentheses): *S. halepense* (Argentina, Australia, China and United States of America); *S.* × *almum* (Argentina, Australia, Ethiopia and United States of America); *S. bicolor* (Argentina, Brazil, China, France, United States of America, and two from an unknown area); *S. vulgare* (unknown); *S. verticilliflorum* (unknown); *S. saccharatum* (China, and

[1] Laboratories may find that alternative DNA extraction techniques work equally well.

three from an unknown area); *S. nitidum* (Australia and China); *S. arundinaceum* (Australia); *S. drummondii* (Democratic Republic of the Congo, Ethiopia, Kenya and Portugal); *S. sudanense* (Argentina and China); *Sorghum* spp. hybrid cv. Silk (Australia); and S. *propinquum* (China). At least ten seeds are needed for each sample.

The PCR primers used in this assay are as described by Zhang *et al.* (2013):

SH1: 5′-AGATTGAGTCTCAGGTGC-3′

SH2: 5′-GAGTCTCAGGGTATGATCT-3′

Each 20 µl amplification reaction contains 2 µl 10× PCR buffer, 0.4 mM dNTPs, 0.25 mM of each primer, 1 U Taq DNA polymerase and 25 ng DNA (made up to volume with ddH_2O). The thermocycler is programmed for 35 cycles of 30 s at 94 °C, 40 s at 55 °C and 80 s at 72 °C. The PCR products are analysed by gel electrophoresis.

The primers produce a diagnostic band of 500 bp, which is found in *S. halepense* samples and some *S.* × *almum* samples from Australia. No bands are produced by *S. bicolor*, *S. vulgare*, *S. verticilliflorum*, *S. saccharatum*, *S. nitidum*, *S. arundinaceum*, *S. drummondii*, *S. sudanense*, *Sorghum* spp. hybrid cv. Silk and *S. propinquum*.

4.2.2 Controls for molecular tests

For the test result obtained to be considered reliable, appropriate controls – which will depend on the type of test used and the level of certainty required – should be considered for each series of nucleic acid isolation and amplification of the target pest or target nucleic acid. For ISSR and SCAR PCR a positive nucleic acid control, a positive extraction control, an internal control and a negative amplification control (no template control) are the minimum controls that should be used.

Positive nucleic acid control. This control is used to monitor the efficiency of the test method (apart from the extraction). Pre-prepared (stored) genomic DNA of *S. halepense* may be used.

Internal control. For ISSR and SCAR PCR, plant internal controls *matK-trnK* or other suitable targets should be incorporated into the protocol to eliminate the possibility of PCR false negatives due to nucleic acid extraction failure or degradation or the presence of PCR inhibitors. Preferably, these internal control primers should be used:

CP3: 5′-ACGAATTCATGGTCCGGTGAAGTGTTCG-3′

CP4: 5′-TAGAATTCCCCGGTTCGCTCGCCGTAC-3′

The length of the PCR product is 750 bp (Zhang *et al.*, 2013). The laboratory should choose an internal control and validate it.

Negative amplification control (no template control). This control is necessary for PCR to rule out false positives due to contamination during preparation of the reaction mixture. PCR-grade water that was used to prepare the reaction mixture is added at the amplification stage.

Positive extraction control. This control is used to ensure that target nucleic acid extracted is of sufficient quantity and quality for PCR.

The positive control should be approximately one-tenth of the amount of DNA extracted.

For PCR, care needs to be taken to avoid cross-contamination due to aerosols from the positive control or from positive samples. The positive control used in the laboratory should be sequenced so that this sequence can be readily compared with sequences obtained from PCR amplicons of the correct size. Alternatively, synthetic positive controls can be made with a known sequence that, again, can be compared with PCR amplicons of the correct size.

Negative extraction control. This control is used to monitor contamination during nucleic acid extraction and/or cross-reaction with other plants. The control comprises nucleic acid that is extracted

from the plant that caused contamination and subsequently amplified. It is recommended that multiple controls be included when large numbers of positive samples are expected.

4.3 Biochemical identification of seeds

The sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) for seed prolamin method of Fang *et al.* (2007) is used to distinguish *S. halepense* from the closely related species *S. bicolor*, *S. sudanense* and *S.* × *almum*. *Hordeum vulgare* is used as a control.

For each sample, at least 10 but preferably 30 mature, plump seeds are ground into a fine powder, and 0.1 g of the powder is transferred to a 1.5 ml microtube. Solvent (600 µl) mixed with the 60% mass fraction of n-propanol, glycol, isopropanol and tert-butanol is added to the sample powder in the tube. The slurry is incubated at 37 °C for 10 h, then is centrifuged at 7 100 *g* for 15 min. The supernatant, which is the prolamin extract, is transferred to a clean tube and stored at 4 °C until it is needed.

Prolamin extract (500 µl) is added to the same volume of cold acetone. The mixture is incubated at 4 °C for 10 min, then is centrifuged at 7 100 *g* for 15 min. The supernatant is discarded, and the prolamin pellet is air-dried at room temperature, then dissolved in 100 µl resuspension buffer containing 6 M urea, 30% glycerine and 25 mM acetic acid.

A 25 µl volume of the final prolamin sample is loaded onto a 15% acid (A)-PAGE gel for a run at 150 V for 4.5 h. The electrophoretic buffer is acetic acid–glycine solution (pH 3.2–3.5). Protein bands are stained with Coomassie Brilliant Blue G-250, then analysed with a gel imaging system.

The prolamins from seeds of different species show different numbers of bands in different (α, β and γ) areas by A-PAGE, as follows (see also Figure 5 for a diagrammatic representation):

- *S. halepense:* one band in the γ area
- *S. bicolor:* three bands in the α area, one band in the β area and two bands in the γ area
- *Sorghum* hybrid (*S. sudanense* × *S. bicolor*): two bands in the β area and two bands in the γ area
- *S. sudanense:* two bands in the γ area
- *S.* × *almum:* no bands.

4.4 Morphological identification of plants

Seeds can be grown for more than 100 days into mature plants for the identification of *S. halepense*. This method allows rhizomes to be one of the determining factors for the identification. Seeds are incubated for seven days on moistened filter papers in Petri dishes (9 mm in diameter) under a 12 h photoperiod at 25 °C. Seedlings with roots and leaves are transplanted into 10 cm diameter plastic pots containing a sterilized soil mix of 1:1:1 sand, soil and peat. The pots with the transplanted seedlings are placed in a greenhouse under natural light and with 28 °C/20 °C day/night temperatures.

There are many resources in the literature on plants and weeds that may be used to identify the family Poaceae, genus *Sorghum* and species *S. halepense*. In this diagnostic protocol the characters used to identify *S. halepense* are from Holm *et al.* (1977) and Flora of China Editorial Committee (2013). Figures 1 and 2 show the morphological characters of *S. halepense*. Additional photos are available at USDA (n.d.a).

Seedling: Coleoptile approximately 13 mm, primary leaves linear, 28 mm × 3 mm; hypocotyl 16–18 mm in length and epicotyl 4–6 mm (Guo and Huang, 1992). Seedlings are the earliest stage at which an identification can be made.

Mature plant: Perennial with vigorous, spreading rhizomes. Culms 0.5–1.5 (–3.0) m tall, 4–6 (–20) mm in diameter; nodes puberulous. Leaf sheaths glabrous; leaf blades linear or linear-lanceolate, (10–) 25–80 (–90) × (0.5–) 1–4 cm, glabrous; ligule 0.5–1 (2–6) mm, glabrous ciliolate membrane.

Inflorescence: Panicle lanceolate to pyramidal in outline, (10–) 20–40 (–55) cm, soft white hairs in basal axil; primary branches solitary or whorled, spreading, lower part bare, upper part branched, the secondary branches tipped by racemes; racemes fragile, composed of (1–) 2–5 spikelet pairs.

Spikelet: Usually in pairs although towards the tip of the inflorescence they may occur in threes; when the spikelet is in pairs, the lower is sessile and perfect with the upper pedicelled, narrow, long and stamen-bearing; when the spikelet is in threes, one is sessile and perfect, the others are pedicelled and staminate. Sessile spikelet elliptic, (3.8–) 4–5 (–6.5) mm; callus obtuse, bearded; lower glume subleathery, often pale yellow or yellowish brown at maturity, shortly pubescent or glabrescent, 5–7-veined, veins distinct in upper part, apex tridenticulate; upper lemma acute and mucronate or bilobed and awned or not; awn 1–1.6 cm. Pedicelled spikelet staminate, narrowly lanceolate, (3.6–) 4.5–7 mm, often violet-purple.

The following keys can be used to discriminate individual plants of *S. halepense* from the five related *Sorghum* species.

4.4.1 Key to the morphological characters of vegetative organs of *Sorghum halepense* and five related species

Based on Kang *et al.* (2000), Sun *et al.* (2002) and Flora of China Editorial Committee (2013).

1. Annual, without rhizome; usually cultivated or occasionally wild **2**

– Perennial, developed rhizome; usually wild or occasionally cultivated **3**

2. Culm base 20–50 mm in diameter ..... ***S. bicolor***

– Culm base 3–9 mm in diameter ..... ***S. sudanense***

3. Rhizome thick and short **4**

– Rhizome thin and developed **5**

4. Culm base 10–30 mm in diameter, node with grey short pubescent, ligule an eciliolate membrane, 0.1–1 mm long, with clear hair in the apex ..... ***S. propinquum***

– Culm base less than 10 mm in diameter, node glabrous, ligule a ciliolate membrane, 2.5–3.5 mm long, without clear hair in the apex ..... ***S. × almum***

5. Culm robust with base approximately 10 mm in diameter, thicker than rhizome; leaf with trichome or shell-like trace on the epidermis ..... ***Sorghum* spp. hybrid cv. Silk**

– Culm fragile with base 4–6 mm in diameter, thinner than rhizome; leaf glabrous ..... ***S. halepense***

4.4.2 Key to the morphological characters of reproductive organs of *Sorghum halepense* and five related species

Based on Flora of China Editorial Committee (2013).

1. Racemes robust and not easily fractured ..... ***S. bicolor***

– Racemes fragile **2**

2. Pedicelled spikelet persistent **3**

– Pedicelled spikelet deciduous **4**

3. Panicle 30–50 cm long, dark magenta; caryopsis deep red–brown ..... ***S. × almum***

– Panicle 15–30 cm long, magenta; caryopsis red–brown ..... ***S. sudanense***

4. Racemes loosely arranged ..... ***Sorghum* spp. hybrid cv. Silk**

– Racemes tightly arranged **5**

5. Panicle ovate; sessile spikelet ovate ..... ***S. propinquum***

– Panicle lanceolate; sessile spikelet elliptic ..... ***S. halepense***

4.5 Cytological identification of plants

Chromosome counts and flow cytometry techniques may be used for the identification of *S. halepense* (Price *et al.*, 2005; Li *et al.*, 2009; Jessup *et al.*, 2012). The chromosome number of *S. halepense* (2n = 4x = 40) is greater than that of four of its relatives: *S. Propinquum* (2n = 2x = 20), *S. sudanense* (2n = 2x = 20), *S. bicolor* (2n = 2x = 20) and flowering *Sorghum* spp. hybrid cv. Silk (2n = 3x = 30). The chromosome number of *S.* × *almum* and non-flowering *Sorghum* spp. hybrid cv. Silk (2n = 4x = 40) is the same as for *S. halepense*.

4.5.1 Chromosome counts

Chromosome counts may be made following the method of Price *et al*. (2005). Root tips (approximately 4 mm long) are removed from plants, treated with an aqueous 0.4% 8-hydroxyquinoline solution for 5 h at room temperature, fixed in 95% ethanol–glacial acetic acid (4:1 v/v), rinsed several times with distilled water, hydrolysed for 5 min in 0.1 M hydrochloric acid, rinsed for 5 min with distilled water and washed for 5 min in citrate buffer (pH 4.5). To digest the cell wall, root tips are treated for 15–50 min at 37 ℃ with aqueous 5% cellulase (pH 4.5) and 1.0% pectolyase Y-23, and rinsed three times with distilled water. Rinsed meristems are placed on a clean glass slide with a drop of ethanol–acetic acid (3:1 v/v), teased apart with a fine-tipped pair of tweezers, and allowed to air-dry at room temperature for two days. The chromosomes are stained with Azure Blue. Chromosomes from two or more root tips of each plant are counted.

Samples with more than 30 chromosomes can be suspected to be *S. halepense*, or *S.* × *almum* or non-flowering *Sorghum* spp. hybrid cv. Silk. *S. propinquum*, *S. sudanense*, *S. bicolor* and flowering *Sorghum* spp. hybrid cv. Silk can be excluded. Furthermore, samples with 40 chromosomes can be identified as *S. halepense* when *S.* × *almum* and non-flowering *Sorghum* spp. hybrid cv. Silk are excluded on the basis of being non-flowering and having a short rhizome.

4.5.2 Flow cytometry

Flow cytometry may be carried out following the method of Li *et al*. (2009) and Jessup *et al*. (2012). Newly expanded leaf material from seedling plants which the suspected *S. halepense* seeds have grown into is collected aseptically, kept on ice, chopped finely using a standard razor blade and macerated in 0.25 ml Galbraith's buffer (pH 7.2) in a Petri dish. The chopped leaves are filtered through a 53 µm nylon mesh. An additional 1.0 ml Galbraith's buffer is added and the material is then strained through a filter into a 2.0 ml microtube. Propidium iodide is added to the microtube to a final volume of 50 µl and the mixture is allowed to incubate for 15 min at 0°C.

The mean fluorescence of nuclei is quantified using a flow cytometer (Coulter Electronics[2]) equipped with a water-cooled laser tuned at 514 nm and 500 mW. Fluorescence at >615 nm is detected with a photomultiplier screened by a long pass filter. The mean 2C DNA content of each target species is calculated by comparing its mean nuclear fluorescence with the mean nuclear fluorescence of an

[2] In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

internal standard. Because of the variation of *Sorghum* DNA content, one of two different internal standards is used to avoid overlap of the standard and target species. One standard, *Arabidopsis thaliana* ecotype Columbia, has a genome size of 157 Mb or 1C = 0.16 pg. The DNA content of *A. thaliana* and *S. bicolo*r Tx623 (2C DNA content = 1.67 pg) is determined from 15 replicates of leaf samples from *S. bicolor* and *A. thaliana* Columbia. At least three replicates for each test sample are analysed to obtain the mean DNA content (Price *et al*., 2005; Jessup *et al*., 2012).

Samples with more than 30 chromosomes can be suspected to be *S. halepense*, or *S.* × *almum* or non-flowering *Sorghum* spp. hybrid cv. Silk. *S. propinquum*, *S. sudanense*, *S. bicolor* and flowering *Sorghum* spp. hybrid cv. Silk can be excluded. Furthermore, samples with 40 chromosomes can be identified as *S. halepense* when *S.* × *almum* and non-flowering *Sorghum* spp. hybrid cv. Silk are excluded on the basis of being non-flowering and having a short rhizome.

4.6 Comparison of the confidence level of the identification methods

The seed identification method based on seed morphology is the preferred and most reliable of the five methods described for the identification of *S. halepense*. Identification based on morphological traits of vegetative organs and sexual reproductive organs of mature plants is also reliable. Molecular and biochemical methods are conditional and limited because they have been based on regional and limited samples of *S. halepense*. If there is a lack of confidence in seed identification, molecular, biochemical, cytological and morphology of mature plant identification methods may be used as complementary methods. A comparison of the confidence level of the identification methods is presented in Table 3.

Table 3. Confidence levels for the identification methods for *Sorghum halepense*

Method		**Sample source**	**Reliability**	**Sample sources in making the methods**
Morphological identification of seeds		Seeds	Reliable	Large number of samples, worldwide
Molecular identification	Inter-simple sequence repeat (ISSR) markers	Seeds or parts of plants	Limited or regional	30 individuals in each sample of six species
	Sequence characterized amplified region (SCAR) markers	Seeds or parts of plants	Limited or regional	65 samples of 12 species from Argentina, Australia, Brazil, China, Democratic Republic of the Congo, Ethiopia, France, Kenya, Portugal, United States
Biochemical identification		Seeds	Limited or regional	Each sample of five *Sorghum* species from involved eight species
Morphological identification of plants		Mature plants	Reliable	Large number of samples, worldwide

Method	Sample source	Reliability	Sample sources in making the methods
Cytological identification	Mature plants	Limited or regional	2-8 individuals from United States of America

5. Records

Records and evidence should be retained as described in section 2.5 of ISPM 27 (*Diagnostic protocols for regulated pests*). In cases where other contracting parties may be affected by the results of the diagnosis, the records and evidence and additional material should be kept for at least one year in a manner that ensures traceability.

6. Contact Points for Further Information

Further information on this protocol can be obtained from:

Weed Research Laboratory, Nanjing Agricultural University, Tongwei Rd 6, Weigang, Nanjing 210095, China (Sheng Qiang; e-mail: qiangs@njau.edu.cn or wrl@njau.edu.cn; tel. and fax: +86 25 84395117).

United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), Plant Protection and Quarantine (PPQ), Seed Examination Facility, Building 308, Room 319, BARC-East, Beltsville, MD 20705, United States of America (Rodney W. Young; e-mail: rodney.w.young@aphis.usda.gov; tel.: +1 301 313 9333; fax: +1 301 504 9840).

Department of Plant Protection, Faculty of Agriculture, Çanakkale Onsekiz Mart University, Çanakkale, Turkey (Ahmet Uludag; e-mail: ahuludag@yahoo.com; tel.: +90 537 578 1211).

A request for a revision to a diagnostic protocol may be submitted by national plant protection organizations (NPPOs), regional plant protection organizations (RPPOs) or Commission on Phytosanitary Measures (CPM) subsidiary bodies through the IPPC Secretariat (ippc@fao.org), which will in turn forward it to the Technical Panel on Diagnostic Protocols (TPDP).

7. Acknowledgements

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8. References

The present annex refers to ISPMs. ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

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9. Figures

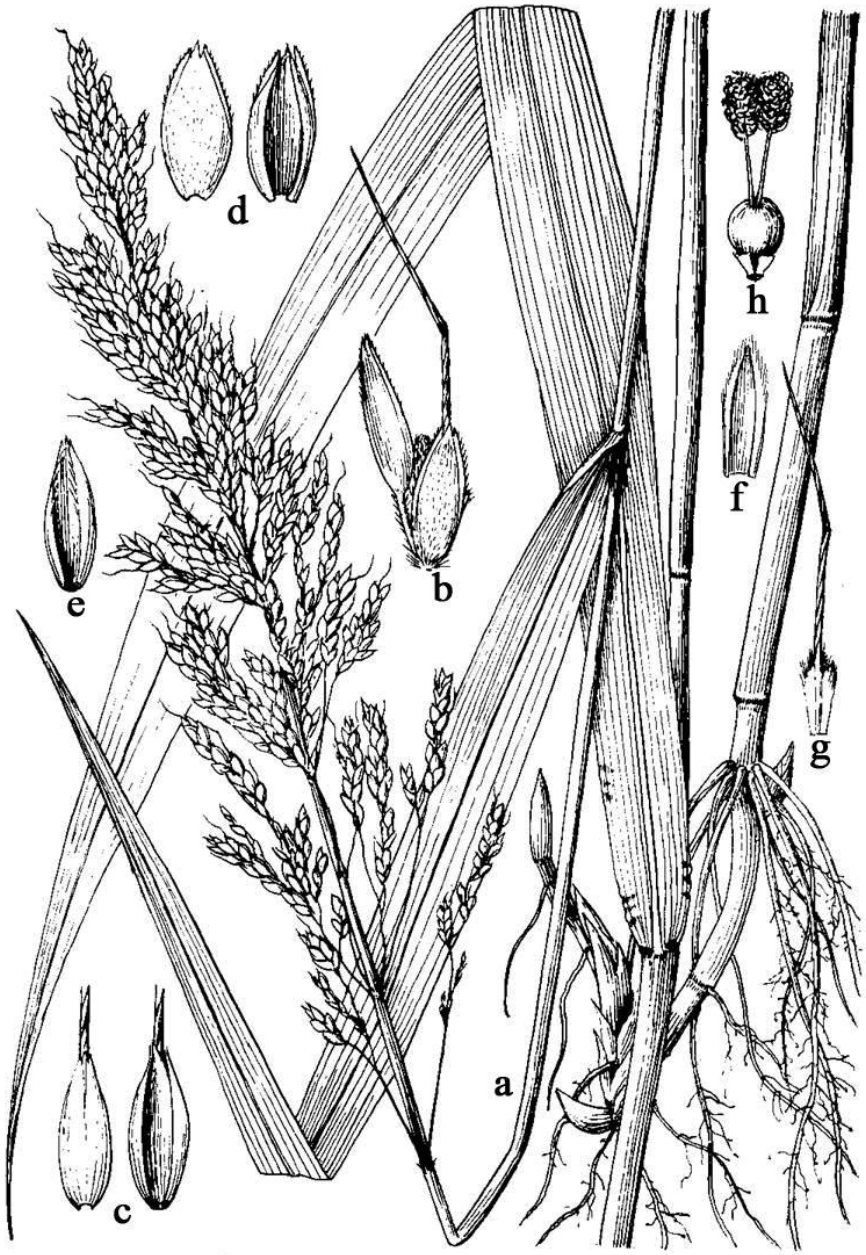

Figure 1. Parts of a *Sorghum halepense* plant: (a) whole plant; (b) spikelet; (c) abaxial and adaxial views of sessile spikelet; (d) lower glume of sessile spikelet; (e) upper glume of sessile spikelet; (f) lower lemma of sessile spikelet; (g) upper lemma of sessile spikelet; and (h) lodicules and pistil.
Source: Flora of China Editorial Committee (1997; plate 28, 1–8).

Figure 2. Morphological characteristics of *Sorghum halepense*: (a) above-ground parts; (b) rhizome; (c) sheath mouth; (d) part of panicle; and (e) perfect spikelet with two pedicelled, staminate spikelets.
Photo courtesy Sheng Qiang, Nanjing Agricultural University, China.

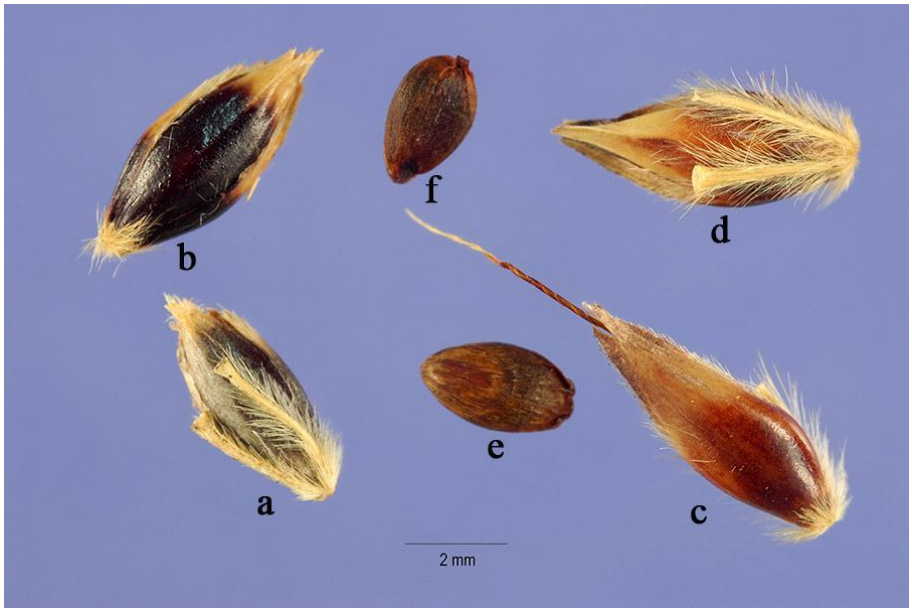

Figure 3. Spikelets and caryopses of *Sorghum halepense*: (a) and (d) adaxial view of sessile spikelet with residual rachilla; (b) abaxial view of sessile spikelet; (c) abaxial view of sessile spikelet with awn; (e) abaxial view of caryopsis; and (f) adaxial view of caryopsis.
Source: United States Department of Agriculture (n.d.b).

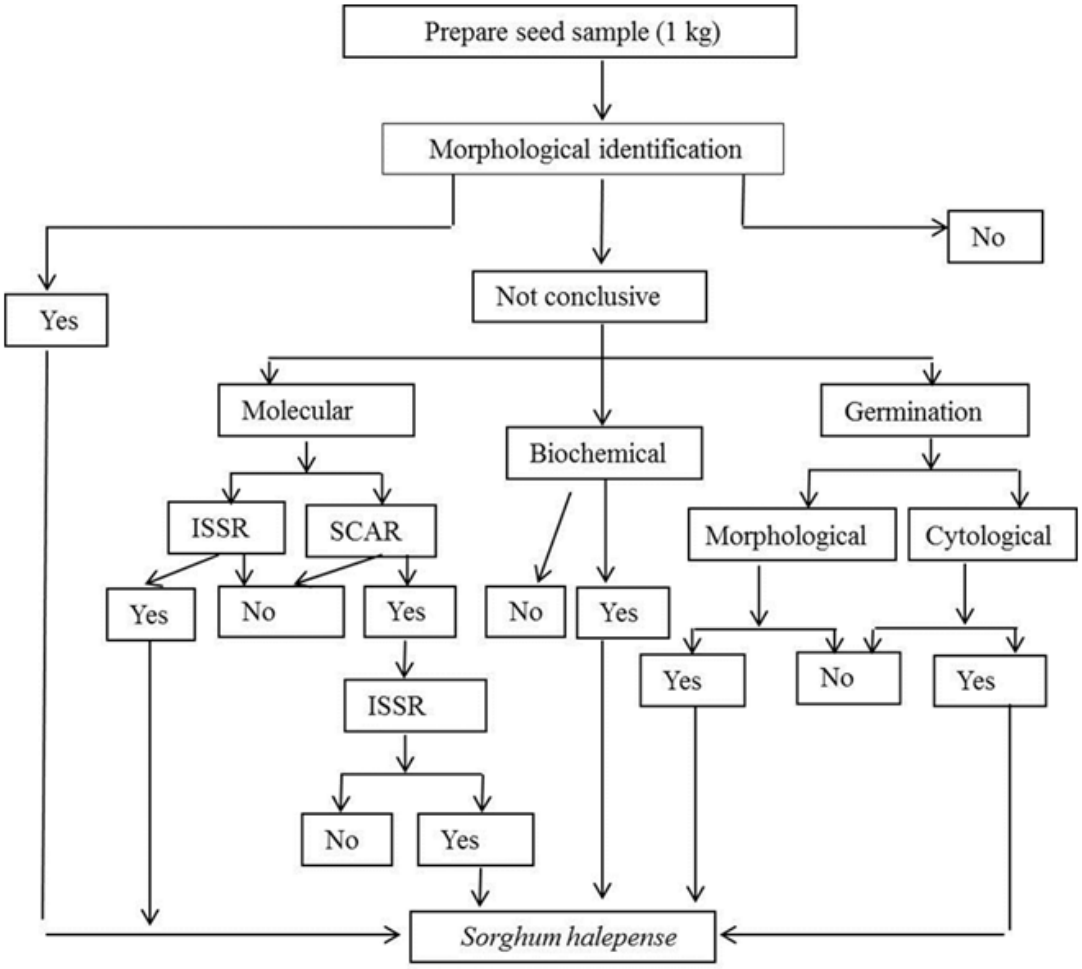

Figure 4. Flow chart for the identification of *Sorghum halepense*.
ISSR, inter-simple sequence repeat; SCAR, sequence characterized amplified region.

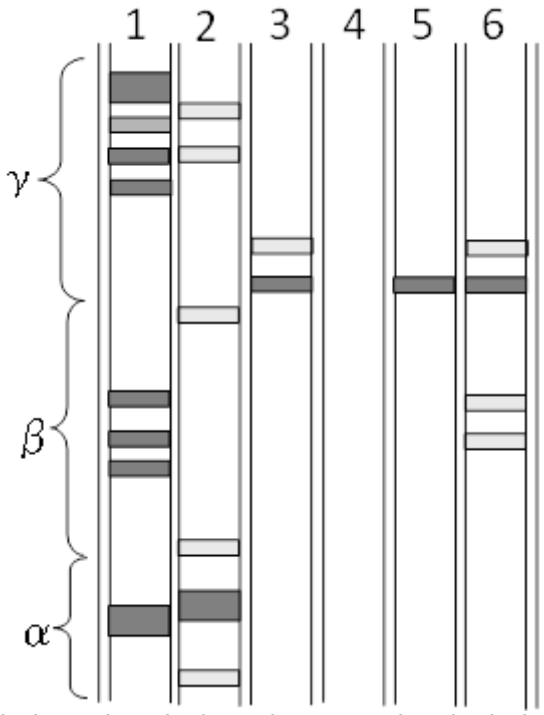

Figure 5. A-polyacrylamide gel electrophoresis (PAGE) pattern of prolamin bands from seeds of different *Sorghum* species: (1) *Hordeum vulgare* (control); (2) *S. bicolor*; (3) *S. sudanense*; (4) *S.* × *almum*; (5) *S. halepense*; and (6) *S. sudanense* × *S. bicolor*.

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IPPC

The International Plant Protection Convention (IPPC) is an international plant health agreement that aims to protect cultivated and wild plants by preventing the introduction and spread of pests. International travel and trade are greater than ever before. As people and commodities move around the world, organisms that present risks to plants travel with them.

Organization

- There are over 180 contracting parties to the IPPC.
- Each contracting party has a national plant protection organization (NPPO) and an Official IPPC contact point.
- Nine regional plant protection organizations (RPPOs) work to facilitate the implementation of the IPPC in countries.
- IPPC liaises with relevant international organizations to help build regional and national capacities.
- The Secretariat is provided by the Food and Agriculture Organization of the United Nations (FAO).

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