Guidelines

For the Conduct of Test for

Distinctiveness, Uniformity and Stability

on

Ashwagandha (*Withania somnifera* (L.) Dunal)

Protection of Plant Varieties and Farmers' Rights Authority (A Statutory Body created by an Act of Parliament)

Government of India, New Delhi

Contents

Ashwagandha (*Withania somnifera* (L.) Dunal)

I. Subject

These test guidelines shall apply to all varieties of Ashwagandha [*Withania somnifera* (L.) Dunal].

II. Seed material required

1. The Protection of Plant Varieties and Farmer's Rights Authority (PPV&FRA) shall decide when, where, and in what quantity of the seed's material is required for testing a variety denomination applied for registration under the Protection of Plant Varieties & Farmers Rights (PPV&FR) Act, 2001. Applicants submitting such seed material from a country other than India shall ensure that all customs and quarantine requirements stipulated under relevant national legislations and regulations are complied with.

2. The minimum quantity of 60 g seeds of varieties by the applicant should be divided equally into 10 packets containing 6 g each.

3. The seeds supplied quality should have the following standards:

(i) Germination: at least 70%

(ii) Purity: > 98% physical purity, highest genetic purity, and uniformity

(iii) Moisture content: shall not exceed 8-9%

4. The seeds supplied shall meet the highest sanitary and phytosanitary standards.

5. The seed material submitted shall not have undergone any chemical and bio-physical treatments unless the competent authority allows or requests such treatment. If it has been treated, full details of the treatment must be given. Certified data on seed germination tests made not more than one month before submission should be provided.

III. Conduct of tests

1. The minimum duration of tests shall normally be at least two independent but similar growing seasons.

2. The test shall normally be conducted at least at two test locations. If any essential characteristics of the variety are not expressed for visual observation at these locations, the variety shall be considered for further examination at another appropriate test site or under special test protocol at the expressed request of the applicant, for which an additional quantity of seeds shall be required.

3. The field test shall be carried out under conditions favoring normal growth and expression of all test characteristics. The size of the plot shall be such that plants or parts of the plant could be removed for measurement and observation without prejudicing the other observations on the standing plants

until the end of the growing period. Each test shall include about 360 plants, in the plot size and planting space specified below across 3 replications. Separate plots for observation and for measurement can only be used if they have been subjected to similar environmental conditions. All the replications shall share similar environmental conditions of the test locations.

4. Test plot design

Plot size	: 3.0 × 2.4 m
Number of rows	: 8
Row length	: 3.0 m
Row to row distance	: 30 cm
Plant to plant distance	: 20 cm
Number of replications	: 3
Expected number of plants	: 120 × 3=360

5. Observations shall not be recorded on plants in border rows.

6. Additional test protocols for special purposes shall be established by the PPV&FR Authority.

IV. Methods and observations

1. The characteristics described in the Table of characteristics (see section VII) shall be used for the testing of candidate varieties and hybrids for their DUS.

2. For the assessment of 'Distinctiveness and Stability', observations shall be made on 90 plants or their parts, which shall be equally divided among three replications (30 plants per replication).

3. For the assessment of the 'Uniformity' of characteristics on the plot as a whole which shall be done by a single visual observation of a group of plants or parts of plants a population standard of 2% with an acceptable probability of at least 98% should be applied. In the case of a sample size of 360 plants, the number of off-types should not exceed 7.

4. For the assessment of all color characteristics of leaf, root, and berry, the Royal Horticultural Society (RHS) color chart 5th Edition 2007 and the latest edition shall be used.

5. Stage of recording observation on specific characteristics will be as follows:

Growth stages	*Decimal Code
Established seedlings	10
Active vegetative stage before flowering	20
Appearance of the first flower	30
50% flowering	40
Fruits fully developed before the color break	60
Ripened fruits	70
Dried berry (at harvest)	80
Fresh Root (at harvest)	90
Dry Root (shade dried)	100

* Total growth period of about 180 days (sowing to harvesting) was converted to decimal scale)

6. All observations on leaf and fruit characteristics will be examined on fully developed mature leaves and fruit clusters on the nodes between the 9^{th} to 11^{th} in the case of indeterminate varieties and between the 5^{th} to 8^{th} node of the main stem from the base in the case of determinate varieties.

7. All observations on dried roots will be done on dried roots with a moisture content of 10-12%.

8. Measurements shall be made in metric units.

V. Grouping of varieties

1. The candidate varieties for DUS testing shall be divided into groups to facilitate the assessment of distinctiveness. Characteristics that are known from experience not to vary or to vary only slightly within a variety and in which their various states are fairly evenly distributed across all varieties in the collection are suitable for grouping purposes.

2. The following characteristics shall be used for grouping Ashwagandha varieties:

i. Plant: Growth habit (Characteristic 1)
ii. Appearance of the first flower (Characteristic 3)
iii. Leaf: Lamina shape (Characteristic 7)
iv. Fruit: Berry color at fruit ripening (Characteristic 16)
v. Seed color (Characteristic 21)
vi. Root: Thickness of bark (Characteristic 27)
vii. Root fracture (Characteristic 29)

VI. Characteristics and symbols

1. To assess Distinctiveness, Uniformity, and Stability, the characteristics and their states as given in the Table of characteristics (Section VII) shall be used.

2. Notes (1 to 9) shall be used to describe the state of each character for the purposes of digital data processing and these notes shall be given against the states of the different characteristics.

3. Legend (*) Characteristics that shall be observed during every growing season on all varieties and always be included in the description of the variety, except when the state of expression of a preceding phenological characteristic or by environmental conditions of the testing region.

(+) See explanations in the Table of Characteristics in Section VII. It is to be noted that for certain characteristics the plant parts on which observations are to be taken are given in the explanation of figure(s) for clarity and not for the color variation.

4. The type of assessment of characteristics indicated in column 7 of the Table of Characteristics is as follows:

MG: Measurement by a single observation on a group of plants or parts of plants

MS: Measurement of a number of individual plants or parts of plants

VG: Visual assessment by a single observation of a group of plants or parts of plants

VS: Visual assessment by observations on individual plants or parts of plants.

VII. Table of Characteristics

Sl. No.	Characteristics	States	Note	Example varieties	Stage of observation	Type of assessment
1 (QL*) +	Plant: Growth habit	Determinate	1	RVA-100	60	VG
		Indeterminate	9	Poshita		
2 (QL*) +	Stem: Growth angle° from the soil surface)	Erect	1	Poshita	30	VG
		Spreading	7	RVA-100, CIM-Pushti		
3 (QN*)	Flower: Appearance of the first flower (days)	Early (<90)	3	RVA-100	30	VG
		Medium (90-115)	5	CIM-Pushti, Poshita		
		Late (>115)	7	Pratap		
4 (QN) +	Leaf: Lamina length (cm)	Short (<7.0)	3	Arka Ashwagandha	40	MS
		Medium (7.0-10.0)	5	RVA-100, NMITLI-101		
		Long (>10.0)	7	CIM-Pushti, Pratap		
5 (QN) +	Leaf: Lamina breadth (cm)	Narrow (<4.0)	3	RVA-100	40	MS
		Medium (4.0-7.0)	5	JA-134, NMITLI-118		
		Broad (>7.0)	7	CIM-Pushti, Pratap		
6 (QL)	Leaf: Colour (RHS colour chart)	Strong yellow-green (144A)	1	Manasa landrace	40	VG
		Moderate yellow-green (146B)	3	Arka Ashwagandha		
		Moderate olive green (147A)	5	Pratap		
7 (QL*) +	Leaf: Shape	Lanceolate	1	RVA-100	40	VG
		Ovate	3	JA-20		
		Obtuse	5	CIM-Pushti, Poshita		
		Deltoid	7	JA-134		
		Elliptic	9	Pratap		
8 (PQ) +	Leaf: Deflection	Flat	1	Arka Ashwagandha, Poshita	40	VG
		Revolute	3	DWS37 (IC623444)		
		Conduplicate	5	CIM-Pushti		
9 (QL*) +	Leaf: Margin	Entire	1	RVA-100, Poshita	40	VG
		Undulate	5	CIM-Pushti		
10 (QL*) +	Leaf: Apex	Acuminate	1	RVA-100	40	VG
		Acute	3	CIM-Pushti		
		Obtuse	5	Poshita		

11 (QL*)	Leaf: Pubescence	Sparse	3	RVA-100	40	VG
		Medium	5	CIM-Pushti		
		Dense	7	Poshita		
12 (QN*) +	Leaf: Petiole length(cm)	Short (<1.75)	3	RVA-100	40	MS
		Medium (1.75 - 2.25)	5	CIM-Pushti		
		Long (>2.25)	7	Pratap		
13 (QN) +	Fruit: Calyx length(cm)	Short (<2.2)	3	RVA-100, Poshita	70	MS
		Long (≥2.2)	7	CIM Pushti		
14 (QN*) +	Fruit: Calyx inflation(cm)	Normal (<1.2)	3	VA-1, Poshita	70	VS
		Inflated (≥1.2)	5	CIM-Pushti		
15 (QN*) +	Fruit: Berry diameter(mm)	Small (<5.6)	3	Pratap	70	MS
		Medium (5.6-7.0)	5	JA-20		
		Large (>7.0)	7	RVA-100		
16 (QL) +	Fruit: Berry colour (RHS colour chart)	Vivid orange yellow (23A)	2	JA-20	70	VG
		Strong orange yellow (28B)	4	Arka Ashwagandha		
		Vivid reddish orange (42A)	6	VA-1, Pratap		
17 (QN) +	Fruit: Number of berries per cluster per axil	Low (<5)	3	Chetak, Poshita	70	MS
		Moderate (5-8)	5	RVA-100		
		High (>8)	7	CIM-Pushti		
18 (QN*)	Days to maturity (days)	Early (<170)	3	RVA-100	80	MG
		Medium (170-190)	5	CIM-Pushti, Poshita		
		Late (>190)	7	Pratap		
19 (QN)	Number of seeds/ berry	Low (<28)	3	RVA-100	80	MS
		Moderate (28-35)	5	JA-20, Pratap		
		High (>35)	7	Arka Ashwagandha		
20 (QL) +	Seed shape	Reniform	3	Poshita	80	VG
		Triangular	7	CIM-Pushti		
21 (QL) +	Seed colour (RHS colour chart)	Light yellow (15D)	3	CIM-Pushti	80	VG
		Vivid yellow (17C)	5	JA-20		
		Strong orangish yellow (26A)	7	VA-1, Pratap		
22 (QN)	Seed test weight (1000 seeds) (g)	Low (<1.85)	3	CIM-Pushti, Pratap	80	MS
		Medium (1.85-2.30)	5	RVA-100, Poshita		
		High (>2.30)	7	Arka Ashwagandha		
23 (QN*)	Plant height (cm)	Short (<60)	1	JA-20	80	MS
		Semi-dwarf (60-90)	5	Arka Ashwagandha		
		Tall (>90)	7	CIM-Pushti, Pratap		

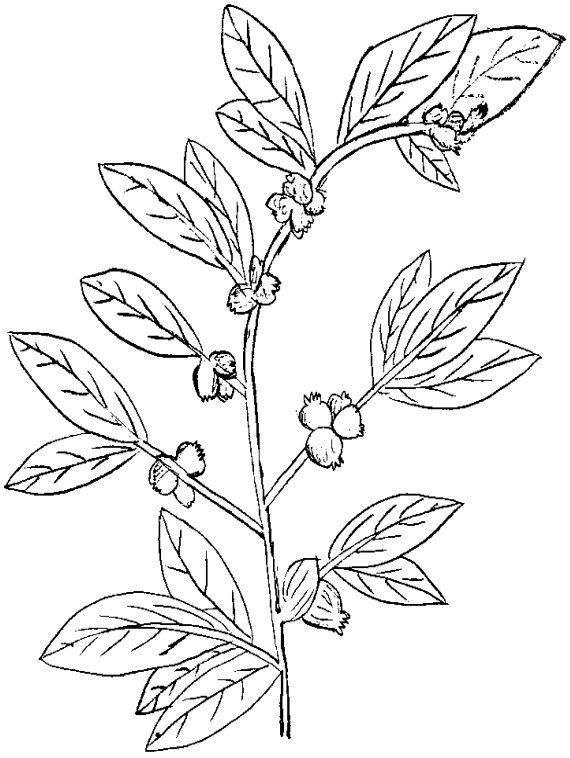
24 (QN*) +	Stem: Inter-nodal distance (cm)	Short (<2.0)	3	JA-20	80	MS
		Medium (2.0-4.0)	5	CIM-Pushti		
		Long (>4.0)	7	Pratap		
25 (QN*) +	Root: Main root length (cm)	Short (<18)	3	JA-20	90	MS
		Medium (18-30)	5	CIM-Pushti		
		Long (>30)	7	Pratap		
26 (QN*) +	Root: Main root diameter (mm)	Thin (<18)	3	JA-20	90	MS
		Moderate (18-25)	5	CIM-Pushti		
		Thick (>25)	7	Pratap		
27 (QN*) +	Root: Thickness of bark (mm)	Thin (<1.75)	3	JA-20	90	MS
		Thick (≥1.75)	7	NMITLI-118		
28 (QL*)	Root (Fresh): Colour (RHS colour chart)	Pale yellow (161D)	1	JA-20	90	VG
		Light yellow (162C)	3	CIM-Pushti		
		Moderate orange yellow (164 C)	5	Pratap		
29 (PQ*) +	Root (Fresh): Brittleness	Brittle	1	JA-20	90	VS
		Break with even fracture	3	CIM Pushti		
		Break with an uneven fracture	5	Pratap		
30 (QN*) +	Leaf total withanolide content (%) on dry weight basis	Low (<0.3)	3	CIM-Pushti	40	MG
		Moderate (0.3-1.0)	5	JA-134, NMITLI-118		
		High (>1.0)	7	Pratap		
31 (QN*) +	Leaf Withaferin-A content (%) on dry weight basis	Low (<0.28)	3	CIM-Pushti	40	MG
		Moderate (0.28-0.70)	5	JA-134, Poshita		
		High (>0.70)	7	Pratap		
32 (PQ*) +	Root polysaccharide content (%)	Low (<15)	3	Pratap	100	MG
		Moderate (15-25)	5	Chetak, NMITLI-101		
		High (>25)	7	JA-20, Poshita		
33 (PQ*) +	Root fibre content (%)	Low (<12.5)	3	JA-20	100	MG
		Moderate (12.5-20)	5	CIM-Pushti, Poshita		
		High (>20)	7	Pratap		
34 (PQ*) +	Root Withanolide-A content (%) on dry weight basis	Low (<0.28)	3	Chetak	100	MG
		Moderate (0.28-0.65)	5	Poshita, NMITLI-118		
		High (>0.65)	7	CIM-Pushti		
35 (PQ*) +	Root total withanolide content (%) on dry weight basis	Low (<0.3)	3	JA-134	100	MG
		Moderate (0.30-0.70)	5	RVA-100, Poshita		
		High (>0.70)	7	CIM-Pushti		

VIII. Explanations on the Table of Characteristics

Characteristic 1. Plant growth habit

Determinate (1): The determinate type (RVA-100) produces a genetically predetermined number of inflorescences on each stem which may be variable among varieties (may be influenced by genotype-environment interaction). The stem ends with an inflorescence and further no lateral shoots are produced. The number of leaves or internodes between two consecutive inflorescent may be irregular from 0-3 within a plant. The stem may terminate variably with 10^{th} inflorescence or 12^{th} inflorescence within a defined range in a semi-determinate (CIM-Pushti) type habit.

Indeterminate (9): In plant growth in which the main stem continues to prolongate from one of the lateral buds and the terminal bud is transformed into the inflorescence, the pattern repeats indefinitely. Leaves and/or internodes are observed between two consecutive inflorescences (Poshita).



1
Determinate

9
Indeterminate

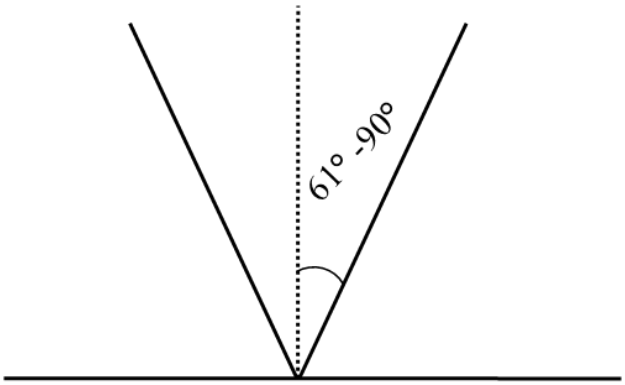
1

Determinate

9

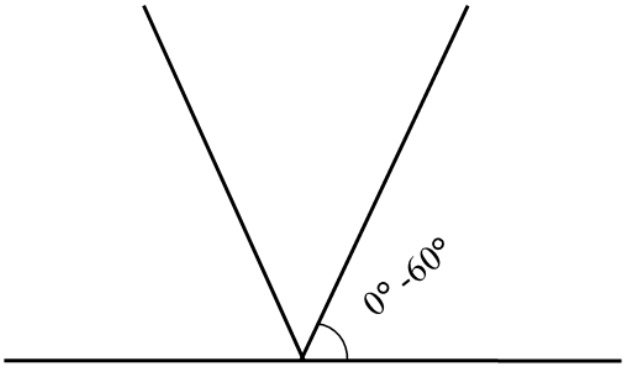
Indeterminate

Characteristic 2. Stem: Growth angle° from soil surface



1

Erect



7

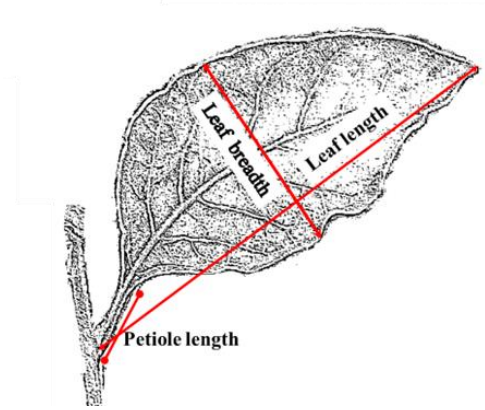
Spreading

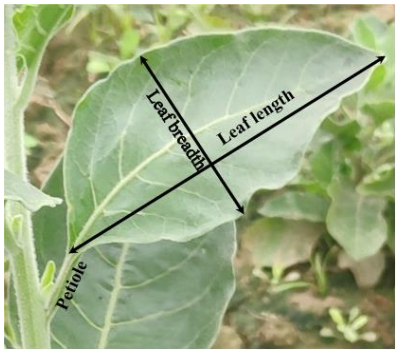
Poshita
(1)

RVA-100
(7)

Characteristics 4, 5 & 12 Leaf: Lamina length, breadth and petiole length (cm)

To be measured on fully developed leaves between the 5th to 8th node of determinate varieties and 9th to 11th node of indeterminate varieties from the base of the main stem from a sample size of 90 leaves. Lamina length from the lamina base to the lamina apex. Breadth shall be taken from the broadest area of the lamina. Petiole length is to be recorded from lamina base to the attachment point on the stem.





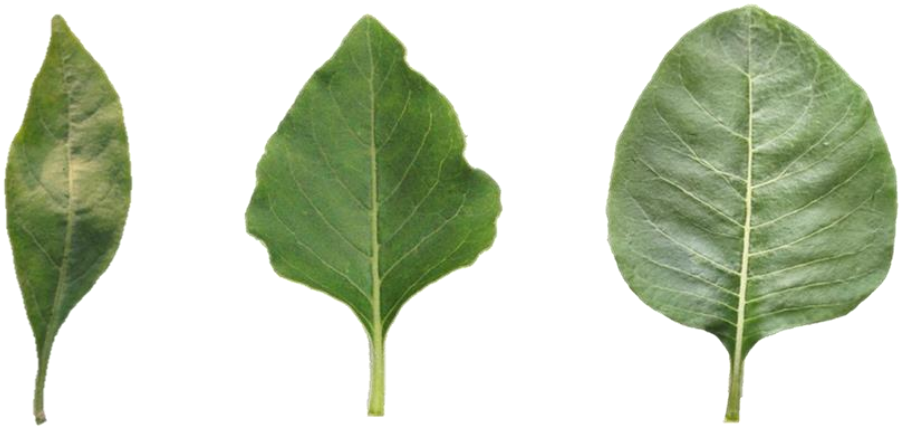
Lamina length

3
Arka Aswagandha

5
RVA-100

7
CIM-Pushti

Lamina breadth

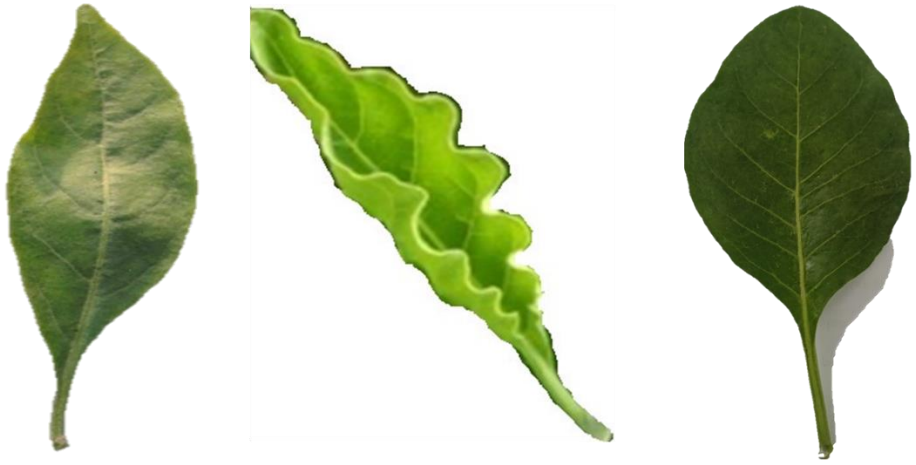


3
RVA-100

5
JA-134

7
Pratap

Petiole length



3
RVA-100

5
CIM-Pushti

7
Pratap

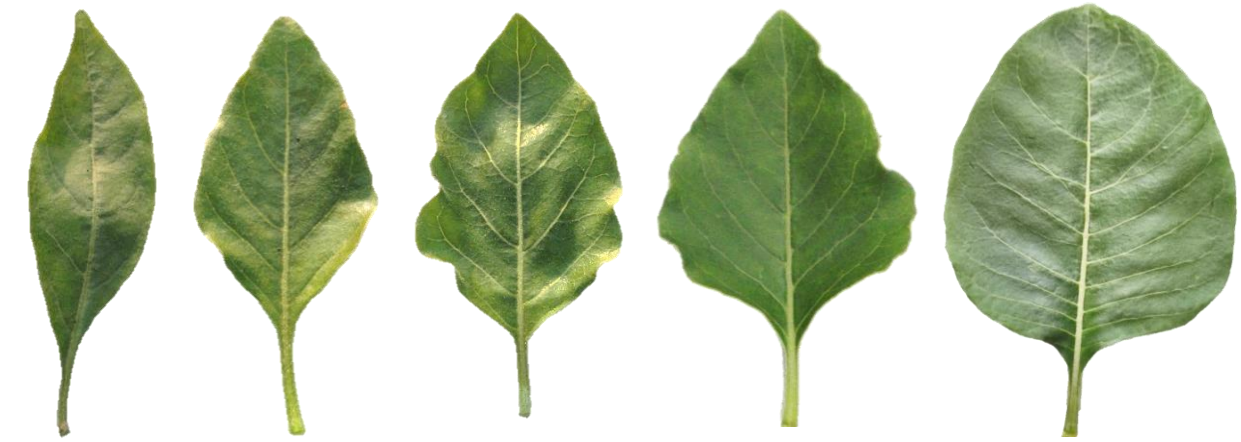
Characteristics 6 Leaf: Colour (RHS colour chart)

1
Strong yellow green
(144A)

3
Moderate yellow green
(146B)

5
Moderate olive green
(147A)

Characteristic 7: Leaf: Lamina shape:



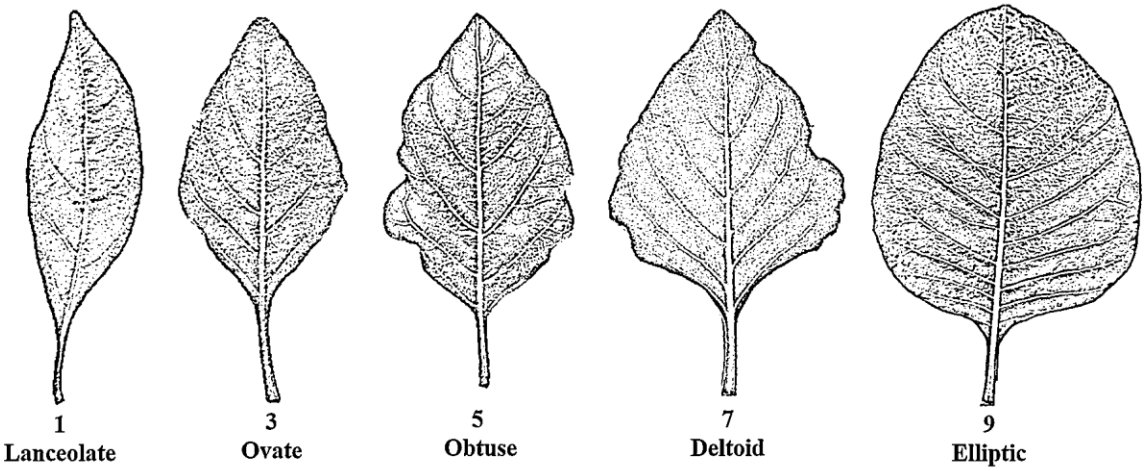
1
Lanceolate

3
Ovate

5
Obtuse

7
Deltoid

9
Elliptic



1
Lanceolate

3
Ovate

5
Obtuse

7
Deltoid

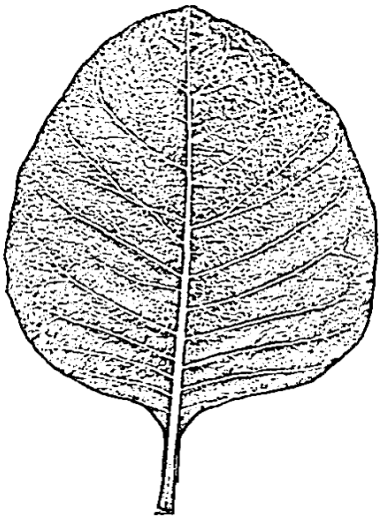
9
Elliptic

Characteristic 8 Leaf: Deflection

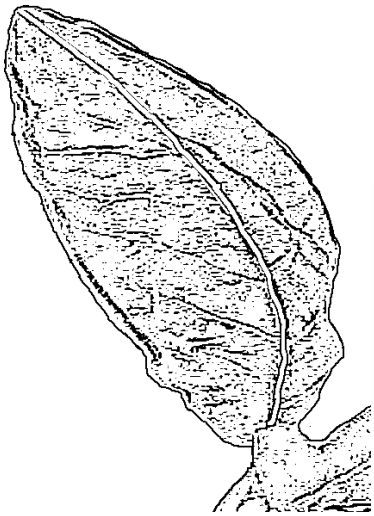
1
Flat

3
Revolute

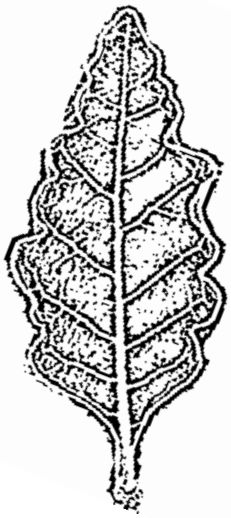
5
Conduplicate



1
Flat



3
Revolute

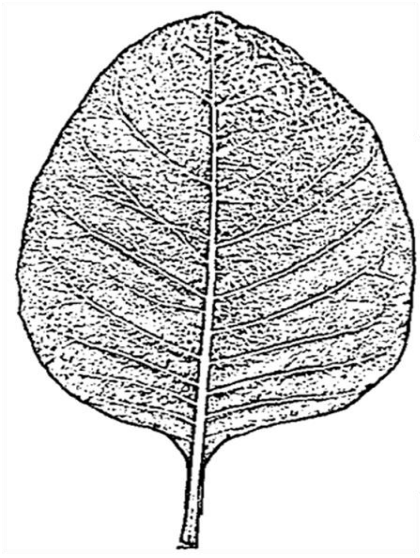


5
Conduplicate

Characteristic 9 Leaf: Lamina Margin

1
Entire

5
Undulate



1
Entire

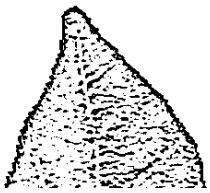
5
Undulate

Characteristic 10 Leaf apex

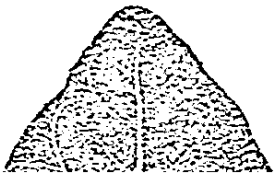
1
Acuminate

3
Acute

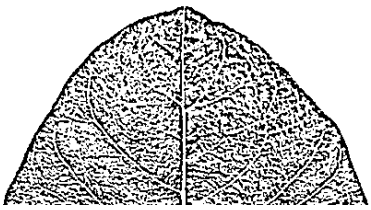
5
Obtuse



1
Acuminate



3
Acute



5
Obtuse

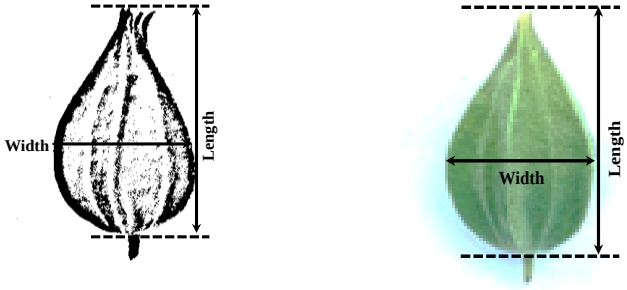
Characteristic 11 Leaf: Pubescence

1
Sparse

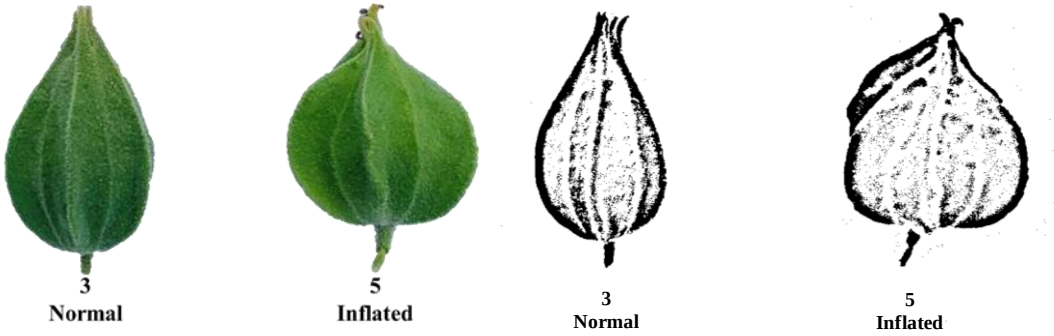
3
Medium

5
Dense

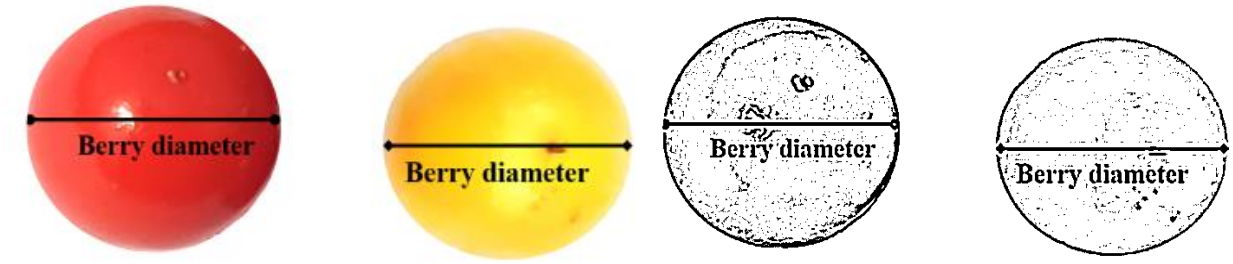
Characteristic 13 Fruit: Calyx length (cm)
To be measured on fully developed fruits between the 5th to 8th node of determinate varieties and 9th to 11th node of indeterminate varieties from the base of the main stem from a sample size of 90 fruits.



Characteristic 14 Fruit: Calyx inflation (cm)
To be measured on fully developed fruits between the 5th to 8th node of determinate varieties and 9th to 11th node of indeterminate varieties from the base of the main stem from a sample size of 90 fruits.



Characteristic15 Fruit: Berry diameter (mm)
To be measured on fully ripened berries between the 5th to 8th node of determinate varieties and 9th to 11th node of indeterminate varieties from the base of the main stem from a sample size of 300 berries.



Characteristic16 Fruit: Berry colour (RHS colour chart)

2
Vivid orange yellow
23A

4
Strong orange yellow
28B

6
Vivid reddish orange
42A

Characteristic 17 Fruit: Number of fruits per cluster per axil

To be measured on at least 3 clusters of fully developed fruits per axil between the 5th to 8th node of determinate varieties and 9th to 11th node of indeterminate varieties from the base of the main stem from a sample size of 90 clusters.

1
Low

3
Moderate

5
High

1
Low

3
Moderate

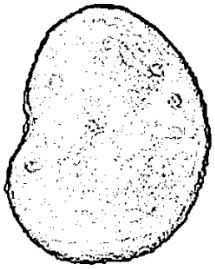
5
High

Characteristic 20 Seed shape

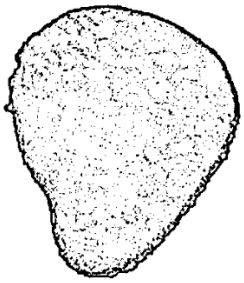
To be observed on seed harvested from fully ripened physiologically mature berries.

3
Reniform

5
Triangular



3
Reniform



5
Triangular

Characteristic 21 Seed color (RHS colour chart)

3
Light yellow
(15D)

5
Vivid yellow
(17D)

7
Strong orangish yellow
(26A)

Characteristic 23 Plant height (cm)

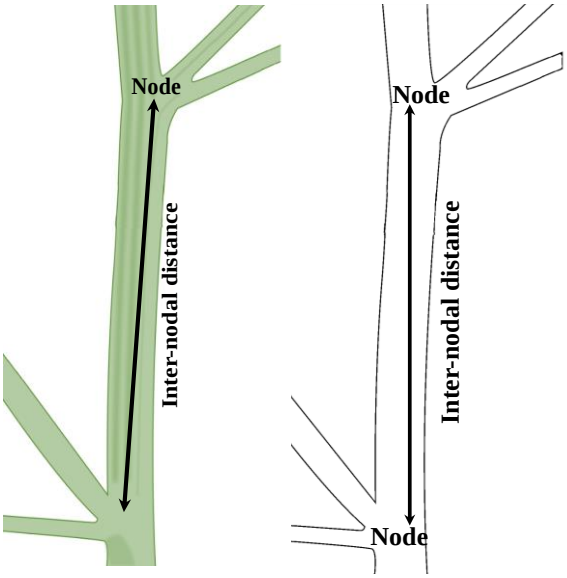
Plant height shall be recorded from the base of the plant to the tallest tip of the longest shoot.

1
JA-20

5
JA-134

9
Pratap

Characteristic 24 Stem: Inter-nodal distance (cm): To be measured from the base of the main stem on three consecutive nodes as defined in section IV (6) and averaging the same**.**



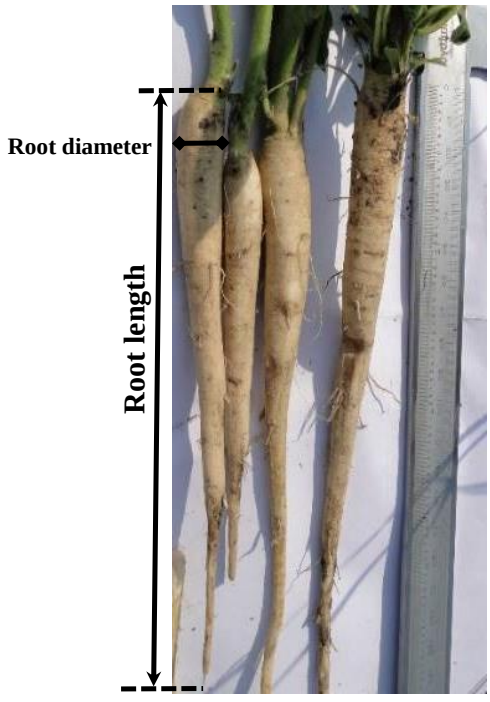
1
CIM-Pushti

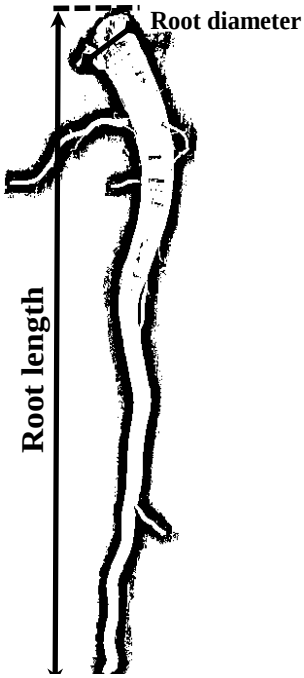
3
JA-20

7
Poshita , Pratap

Characteristics 25 and 26 Root: Main root length (cm) and root diameter (mm)

At the widest portion on root upper part from a sample size of 30 roots just after root digging.





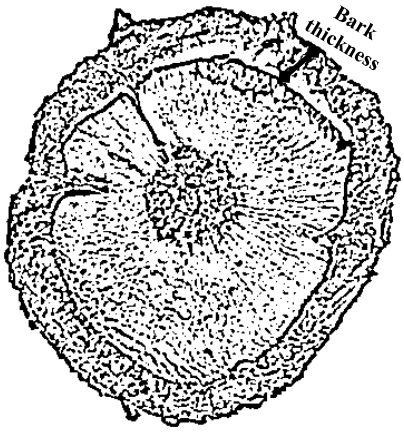
3
RVA-100, JA-20

5
CIM-Pushti, Chetak

7
Poshita

Characteristic 27. Root bark thickness (mm)

To be recorded at the periderm of root bark from a sample size of 30 roots just after root digging.



3
JA-20

5
CIM-Pushti

7
NMITLI-118

Characteristic 28 Root: Outer colour (RHS colour chart)

1
Pale yellow
(161D)

2
Light yellow
(162C)

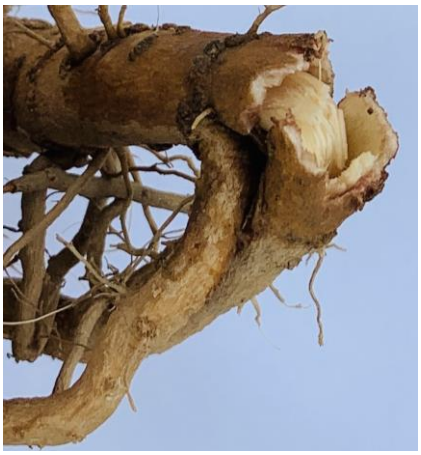
3
Moderate orange yellow
(164C)

Characteristic 29 Root brittleness

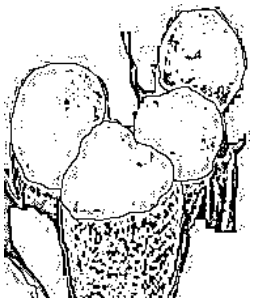
To be recorded just after root digging from a sample size of 30 roots on the slight force.

1
Brittle

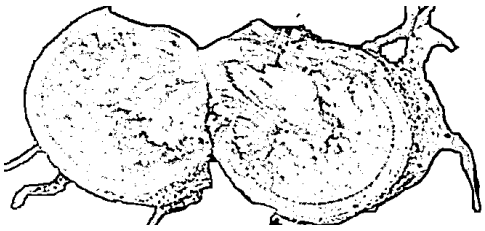
3
Break with even fracture



7
Break with un-even fracture



1
Brittle



3
Break with even fracture

7
Break with un-even fracture

Characteristic 33: Root Polysaccharide content (Hodge and Hofreiter,1962)

Shade-dried homogenized root powder (0.2 g) was treated with 80% alcohol to remove sugars till the washing do not give color with anthrone reagent. The well-dried residue was extracted twice at 0ºC for 20 minutes with 52% perchloric acid and the supernatant was pooled to make up to 100 ml. Standards were prepared by taking 0.2,0.4,0.6,0.8 and 1 ml in each tube with water. Anthrone reagent 4ml each was used for each standard and heated at 100 ºC for eight minutes, cooled by chilling and OD was taken at 630nm. Using a regression curve, glucose content was estimated. Polysaccharide content was estimated by multiplying it by a factor of 0.9.

Characteristic 34: Root Fiber content

Shade-dried homogenized root powder (2 g) was extracted with 200ml of sulphuric acid for 30 min with bumping chips and filtered through a muslin cloth. The residue was washed with boiling water until the washing are no longer acidic. Further, the residue was washed with 200ml of sodium hydroxide solutions for 30 minutes. The residue was filter through muslin cloth and washed with 1.25% H_2SO_4(25ml) and thrice with water(50ml) and finally with alcohol(25ml). The residue was incinerated in a pre-weighed crucible(W1) at 130±2 ºC for 2h, cooled in a desiccator and weighed(W2)it was further ignited at 600ºC ±15ºC for 30 minutes, cooled in a desiccator and reweighed(W3). Crude fiber (%) was estimated as Loss in weight on ignition[(W2-W1)- (W3-W1)] *100/Weight of the samples(Maynard AJ,1970)

Characteristics 30-32 & 35-38 : Withanolide content estimation (Ayurvedic Pharmacopeia of India, 2016)

Shade-dried tissue(leaf /root) (2.0 g) was finely pulverized in a grinder and extracted thrice with 50ml of methanol-water(80:20)v/v) at room temperature by sonication for 20 minutes at 50C. The supernatant was collected, and concentrated through Rotavapour (Bucchi) to a dry powder. A sample of 10 mg of the dry powder was dissolved in HPLC grade methanol(1.0ml) filtered (through 33mm,0.22um, Millex GV) and subjected to analysis on HPLC (WATERS, Model e2695, Milford, USA) and separations were achieved using a Phenomenex Luna reverse phase column C18(250mm x4.6mm x 5um), subjected to a binary gradient elution. The mobile phase was filtered and degassed a gradient mixture of phosphate buffer and acetonitrile with 10ul injection volume at a flow rate of 1.5ml/min and UV detection at 227nm. Reference standards were obtained from ChromaDex. Withanolide glycosides (Withanoside IV and Withanoside V) and withanolide aglycones (Withaferin-A, 12-deoxywithastromonolide, Withanolide-A, Withanolide-B and Withanone) were estimated individually and the sum of these yielded total withanolides. The two solvents used for the analysis consisted of containing Phosphate buffer(A) and Acetonitrile (B). Gradient programming of the solvent system was carried out at 27 ºC (API, 2016. The analysis was carried at a wavelength scan range of 190-400 nm at 227nm using Waters Empower software.

IX **Literature:**

1. The Ayurvedic Pharmacopeia of India Part I Volume VIII. (2016). Pharmacopoeia Commission for Indian Medicinal & Homeopathy. Ministry of AYUSH, Government of India.p28-38.

2. Hodge JE and Hofreiter BT (1962) In: Methods in Carbohydrate Chemistry (Eds Whistler, RL and Be Miller, JN) Academic Press New York.

3. Maynard, AJ (Ed.)(1970) Methods in Food Analysis, Academic Press New York p176**.**

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