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Registration of 'CIM-Pushti': Withanolide-A rich, leaf blight tolerant high yielding variety of ashwagandha (*Withania somnifera*) with good root textural quality

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ABSTRACT

Ashwagandha (*Withania somnifera*) *is an Indian origin, dryland medicinal plant sought across the world as a potent rejuvenative adaptogen owing to its various pharmacological properties. The roots are the most frequently used plant part of Ashwagandha since ancient India Vedic era, as the tissue is enriched with Withanolide A that imparts neuroprotection, mitigates stress and helps to increase brain health. Under a Withanolide targeted breeding program at CSIR-CIMAP, a new variety 'CIM-Pushti' has been developed through intra-specific hybridization between 'Nagouri' X 'Kashmiri', the two major ecotypes prevalent in India. The new variety combines the root quality of Nagouri type (cultivated and annual type) and biomass yield of Kashmiri type (wild, perennial type) in one genotype background. The characteristics of the new variety includes highly wavy, sub-coriaceous, light green leaves with semi erect, strong culm and yellow orange mature berries. Roots are whitish cream with non-separable rind, fine pulverisable, brittle roots with high starch to fibre ratio and Withanolide-A content averaging 0.713mg/g dry weight basis. It is also high in Withanolide- B(0.460mg/g dry weight basis) and negligible presence of Withanone. It matures in 168-178 days producing average yield of 9-10 quintal roots per hectare. It is also tolerant to leaf blight caused by Alternaria alternata. The variety is suitable for cultivation in the Indian agro-climatic zones (VIII,XIII & XIV) i.e. Central Plateau, Western dry region and Gujarat planes regions of India.*

INTRODUCTION

Ashwagandha (*Withania somnifera*, Family: Solanaceae), is the chief natural source of withanolides, the life strengthening adaptogens. It has diverse pharmacological potential owing to its anti-inflammatory, immuno-modulatory, anti-tumour, anti-neurodegenerative properties. It finds its oldest mention in Atherveda, evident from the teachings of Scholar Punarvastu Attreya, who taught medicine at Taxilla University (1000BC) [1,8].

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As per the Ayurveda texts, it is the major component of various formulations of Ayurveda, Siddha and Unani (ASU) traditional medicine. Federal Food, Drug and Cosmetic Act of USA (FDA, US) categorizes it under "generally regarded as safe" (GRAS) group of medicinal plant hence, it holds a wider acceptance in Asian, American as well as European herbal market.

Ashawagandha root extract, which is enriched with Withanolide-A being synthesized *de novo* [18] owes the properties of modulating inflammation, neurotic regeneration, synaptic reconstruction and thus serves as a nerve tonic and nootropic agent [2,4,17]. It can inhibit the glutamate- mediated excitotoxicity of N-methyl D aspartate receptors through allosteric mode and thus is a potential drug for the cure of multi neurodegenerative disorders like Alzheimer's disease, Parkinson's disease, Huntington disease etc.[10,11,16].

So far five varieties of *W. somnifera* have been released from CSIR-CIMAP in previous years i.e. Poshita (2001), NMITLI-118 (2009), Chetak (2011), Pratap (2011), and NMITLI-101(2015).Under ICAR aegis ACRIP have released Jawahar Asgand 20 (1989), Jawahar Asgand 134 (1998) developed at Jawaharlal Nehru Krishi Vishwavidyalaya, Mandsaur and RVA 100 (2011) from Rajmata Vijayaraje Scindia Krishi Vishwavidyalaya (RVSKVV). Ashwagandha varieties so far released in India are indigenous selections in the accessions collected across India and hence carry the feature of only one ecotype out of the two most widely found in India except RVA100. Poshita, NMITLI-118, Pratap and NMITLI-101 are tall, red berry, perennial type where as Jawahar Asgand20, Jawahar Asgand 134, RVA100 and Chetak are dwarf, yellow-orange berry, annual type varieties of Nagouri ecotype. Nagoure type Aswagandha is majorly cultivated in the regions of Shriganganagar, Hanumangarh, Churu, Jhunjhunu, Kota, Boondi, Bara, Jhalawar, Tonk, Sawaimadhopur & Udaipur in Rajasthan, Neemuch, Mandsaur in Madhya Pradesh and parts of Gujarat, Uttar Pradesh, and Maharashtra. It fetches premium market price in the international herbal market, and is exported from India.

The demand of the quality root of Ashwagandha as raw material in the form of cut root, root powder, medicant, dietary supplement, nutraceutical and medicine (Rasayana) in AYUSH systems of medicines is rapidly increasing across the world. As per the Spinscan Naturals, sales of products containing Ashwagandha increased from January 2015 to January 2016 by 63% valuing around US$13 million in US alone [22]. Cultivation of Nagouri Ashwagandha varieties inspite of producing quality root is restrained by productivity, Withanolide-A content [11,17,19] or resistance to leaf blight caused by *Alternaria alternata* which causes major yield losses at post anthesis stage [20]. To meet the growing demands of estimated 10,000 tons roots against the current production of 4000 tons for catering the requirement of nutraceutical and pharmaceutical industries for bioactive withanolides through *in planta* production, a withanolides targeted breeding programme was undertaken at CSIR-CIMAP [3,5-7]. The proposed plant variety has been generated through intra-specific hybridization and intensive metabolite targeted selections and root quality phenotyping in the bi-parental population, combining the root quality of Nagouri type and biomass yield of Kashmiri type and withanolide A content with tolerance to *Alternaria alternata* in one genotype background. The breeding strategies applied for developing this new variety 'CIM-Pushti' is described here.

MATERIALS AND METHODS

Plant Material

Under the Withanolide targeted breeding program at CSIR-CIMAP, a set of 131 accessions were characterized for molecular, phenomic and phytochemical parameters for the years 2007 to 2011 and CWS 11 & CWS 7 was chosen as most diverse potential parents [15]. An intra-specific bi-parental population (CWS11 x CWS7 F_2:6) was developed (2011-2018) and raised in augmented complete block design(2011-2015), with 30x20 cm row-to plant spacing following standard agronomic and cultural practices at CSIR-CIMAP research farm Lucknow. Harvesting of the crop was

conducted at 75% maturity achieved at 180-210 days after transplanting. Traits phenotyped were: leaf length (cm), leaf breadth (cm), leaf shape (leaf length /leaf breadth), internodal distance (cm), growth habit, leaf colour (as per RHS code), plant height (cm), primary branching, secondary branching, calyx length, number of berries per cluster, inflations of calyx (cm), berry size (mm), berry colour(as per RHS code), seeds per berry, thousand seed weight, root length(cm), root colour(as per RHS code), root fracture, fresh root weight (g), dry root weight(g) and herbage yield. Total population size was maintained to be at-least 3000 plants in all generations for effective selection.

Withanolides Profiling

All solvents and reagents for the chemical analysis were of analytical or HPLC grade unless otherwise specified and were procured from Merck India. The standards Withaferrin-A, Withanolide-A, 12 deoxy-withastramonolide, Withanolide B and Withanone were procured from Chromdex (Irvine CA). The leaves and washed roots were shade dried completely, finely pulverized and were used for estimation of total withanolides(%), Withaferin-A, Withanolide-A, 12–Deoxywithastramonolide, Withanolide B and Withanone as described earlier [9, 21].

RESULTS AND DISCUSSION

Pedigree and genetic development of proposed variety

Specified Withanolide targeted breeding programme was undertaken at CSIR-CIMAP. The present work is based on initial three years of (2007-2010) intensive screening for natural variation in phenome, and withanolide content of 131 accessions of *Withania* germplasm [15]. Genetic structure of this natural population was elucidated through microsatellite and semi arbitrary markers like ISSR and URP markers to select diverse elite lines as breeding parents. Based on molecular, phenomic and phytochemical characterization two distinct accessions were selected for intra-specific hybridization. Recipient parent CSW11 was collected from Nagaur (Rajasthan) and is short (30cm). Its root is whitish cream, non-separable rind, fine powderable with soft tuberous starchy texture, which breaks with even fracture (preferred by pharmaceutical industry). It has 5-7 berries at a node which turns yellow on maturity, small light green sub coriaceous leaves with wavy margin, inconspicuous veins and lax branching and high withanolide A content. Donar parent CWS 7 is a accession collected from Patna,was rich in withanolide A content, tall (1.0-1.5 m), heavily branched, high biomass, dark green sharply acute leaves, 12-15 berries at a node which turns red at maturity and long brown root with prominent lateral branching, woody texture, easily separable rind and fibrous powder. ~300 reciprocal F_1 hybrids were generated and QTLs were introgressed in *Withania somnifera* hybrids in the desirable combinations under this *Withania* genetic improvement effort [7,12]. Heterosis was observed for earliness, short height, good root quality, berry size along with root length and content of Withaferrin A, Withanolide A and total root alkaloids in various recombination's [4]. Promising stable recombinant breeding lines with reference to high Withanolide-A, Withaferin-A, plant height, root quality, root starch to fibre ratio, berry colour, branches and branching were generated and subjected for generation advancement with selection pressure on high Withanolide A content and good textural quality root with even fracture with yellow orange berry and dry root yield.

Preliminary Yield Trial (PYT, 2014-15)

Around 300 promising lines (CWS11 x CWS7 F_2:5) were screened at PYT station trial in the year 2014-15 against its parents, local annual check Chetak and three perennial type outgroup checks Pratap, Poshita and NMITILI-118 [14]. Evaluations of plant progeny with three lines each with row to plant spacing of 30x25cm in Augmented block design was conducted as per standard agronomic and agricultural practices. Based on Withanolide Content A, root fracture and high herbage and root yield, 24 entries were selected for initial evaluation trial.

Initial Evaluation Trial (IET, 2015-16)

Twenty four selected entries (CWS11YxCWS7 F_2:6) were screened at IET station trial in year 2015-16 against one local annual check Chetak and three outgroup checks Perennial type Pratap, Poshita and NMITILI-118 in RCBD with Net Plot Size 1.5x2.5m2; spacing30x25 with two replications as per standard agronomic and agricultural practices(Table1). Based on Withanolide Content A, root fracture and high herbage and root yield, thirteen entries were selected for advance varietal trial.

Advance variety trial, (AVT) evaluation (2016-17)

Thirteen advance breeding lines (CWS11xCWS7 $F_{2:7}$) with orange/yellow berry, high withanolide content, annual type, semi-dwarf stature were evaluated against three national checks(JA20,RVA100 JA134), one local check (Chetak) and two outgroup checks(Poshita and NMITLI-118) for agronomic performance. Advance variety trial was conducted in replicated block design with three replications with net plot size 3x2.7m2; spacing 25x15cm as per standard agronomic and agricultural practices (Table 2) [3, 13].

Statement of distinction

The unique feature for identification of the 'CIM Pushti' are: undulated, sub-coracious green leaves, with strong, semi erect culm with culm angle at base around 45-55°, semi dwarf (Figure1), mature berries yellow-orange colored (RHS code 23-28A). Root is cream coloured, smooth, 21-25 cm long (Figure2A) with even fracture and high starch to fiber ratio (Fig2C) and it matures in 165-185 days from DAS. Withanolide Acontent is around 0.713mg /g of dry weight basis. It is also high in Withanolide B content.(0.460mg/g dry weight basis) and lacks Withanone as compared to other Nagour varieties.Withanone is anticancerous in nature,and its large amount may be toxic if used as a nutraceutical supplement(Fig2 D and E). Average dry root yield is around 10.09 quintal per hectare and tolerant to leaf blight caused by *Alternaria alternata* (Table 3, Fig.1). To meet the distinctness

Fig. 1: A field view of CIM Pushti

and novelty criterion, CIM Pushti was compared with JA20, RVA 100, Chetak, Poshita NMITLI-118 and could be differentiated uniquely by 22 in house generated microsatellite markers. A representative four unique markers profile with WATG403.1, WATG403.2, WGA805, WGA559.2 marker profile is shown in Fig 2B. As per UPGMA analysis using DICE as genetic similarity coefficient at 0.62, CIM Pushti was distinctly differentiated from Nagour(JA20, RVA100) as well as wild, perennial varieties (NMITLI-118, Poshita)(Fig 3).

It is estimated that 'CIM Pushti' will generate at least 30% extra root yield in terms of dried biomass in comparison to existing Nagouri type varieties cultivated by farmers and an additional 10% for leaf herbage which will generate an additional income of Rs 52,500/-per hectare as an additional return to the farmers. Thus farmers can obtain a net income of Rs 1,50,000/- from root and Rs. 15,000/- from sale of leaves from the proposed variety within a period of 6 months against a very low inputs of Rs. 40000/ha.In India about 60% of the cultivable area is under rainfed agriculture. Successive years of deficit/delayed rainfall and/or failure of monsoon have further affected such area significantly. ''CIM Pushti" has the potential for greening the dry land zones, making the wastelands productive. It can be cultivated both as a rainfed and irrigated crop suitable for cultivation in the Indian agroclimatic zones (VIII, XIII & XIV) i.e

TABLE 1: Performance of 24 selected advance breeding lines in Initial Evaluation Trial (2015-16).

Entry	ID	LWLD	RWLD	WA	DTM	PH	BM	RL	RD	FRW	SY	RY	RF*
A3-4-5	E1	17.7	23.6	0.031	184	103	407	35.3	1.4	86.8	16.28	3.472	1
H5-3-14-1	E2	11.7	26.5	0.782	174	104	228	60.3	1.4	68.8	9.12	2.752	1
C3-3-1	E3	21.7	13.2	0.605	192	111	650	32.2	1.3	53.9	26	2.156	2
D1-2-4	E4	22.3	27.3	0.069	190	91	680	26	1	85	27.2	3.4	1
D2-11-11	E5	18.6	64.5	0.398	187	84	720	28	2	90	28.8	3.6	2
D2-11-11	E5	19.2	65.2	0.397	186	85	721	27	1.8	92	27.804	3.4605	2
D3-9-4	E6	16.7	32.3	0.1761	192	88	251	27.7	1.2	57.9	10.04	2.316	2
D3-7-3	E7	7.8	13.1	0.585	192	89	800	24.3	1.7	48.6	32	1.944	3
D5-9-15	E8	27	57.9	0.129	187	91	600	22	1.6	60	24	2.4	1
D6-8-7	E9	26.6	46.8	0.02	189	89	1200	22.6	1.3	48.8	48	1.952	3
E1-10-2	E10	32.5	25	0.12	182	94	403	32.7	2	198	16.12	7.92	2
A7-13-1	E11	23.3	27.1	0.096	184	101	416	29	1.5	94.6	16.64	3.784	2
C3-4-3	E12	15.8	36	0.032	195	112	275	27.3	1.6	63.3	11	2.532	2
D2-2-7	E13	16.9	21.4	0.444	190	97	10.25	24	1.7	66.9	4.1	2.676	1
D2-12-3	E14	24	44.1	0.346	188	85	550	29	1.5	70	22	2.8	1
H6-3-10	E15	25.3	44.2	0.08	176	117	1072	35.1	2.4	277	42.88	11.08	3
H6-1-14	E16	27.4	38.4	0.229	175	125	1780	32.4	2.1	338	71.2	13.52	3
E4-15-1	E17	13.86	17.6	0.018	179	120	765	28.1	2.2	170	30.6	6.8	2
E3-5-1	E18	32	42.11	0.723	185	71.5	560	35	1.7	119	22.4	4.76	1
D6-1-15	E19	30.01	56.48	0.13	190	59	480	20	0.8	46	19.2	3.4	1
C3-2-7	E20	15.4	52.3	0.201	189	61	670	28	1.4	84	26.8	3.36	1
C5-5-1	E21	20.6	45	0.085	180	61	760	25	1.8	108	30.4	6.32	1
E4-2-1	E22	18.26	68.4	0.17	188	70	690	30	1.5	53	27.6	3.12	1
D5-13-1	E23	57.14	90.351	0.792	184	53	760	29	1.3	116	30.4	4.64	1
E3-9-2	E24	15	26.8	0.128	194	47	730	25	1.4	60	29.2	2.4	1
CHETAK	C1	20.135	42.325	0.134	189	124	450	33.4	1.6	120	18	4.8	1
PRATAP	C2	21.134	38.242	0.12	184	124	1050	33.4	1.6	312	42	12.48	3
POSHITA	C3	20.123	37.651	0.119	172	109	420	28.4	1.4	230	16.8	9.2	3
NIM	C4	23.341	42.456	0.145	184	127	792	42.6	1.7	290	31.68	11.6	3
SE		1.89	2.34	0.03	0.82	3.1	45.68	1.01	0.04	11.69	1.78	2.07	
CD5%		2.77	3.46	0.033	3.97	3.6	23.34	3.05	0.33	5.62	2.96	0.74	
CV%		9.33	1.98	3.04	0.48	0.871	0.812	2.26	4.62	1.04	2.59	3.3	

Net Plot Size 1.5x2.5m2; spacing30x25
LWLD, RWLD & WA is described in mg/g
SY&RY is described in Kg/plot
*Root Fracture 1: brittle and even (desirable by consumer); 2: uneven and less brittle; 3: bend but do not break (undesired by consumer)

Central Plateau, Western dry region and Gujarat planes regions of India.'CIM Pushti' is a high yielder with more withanolide-A content, yellow berry, good root textural quality which promises to fetch internationally higher price and demands less resources for its cultivation. If taken up by industry, an economic uniform raw drug resource may reduce the cost of Ashwagandha based drugs products. Ashwagandha farmers will be directly benefited for the higher price of their produce. This high yielding variety will also reduce the uprooting of this natural resource from the forests.

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Table 2: Performance of entries (13) against six checks in advance variety trial I (2016-17)

LINE	ENTRY	WA	RWLD	LWLD	S.F	DTM	LL	LB	NTB	PH	BM	FRW	RL	RD	DH	DR
E3-5-1	E1	0.79	48.18	19.82	1.23	181.74	7.39	3.64	23.88	68.59	236.03	37.84	24.81	2.28	629.42	10.09
D1-2-4	E2	0.07	33.25	13.57	1.22	179.04	9.83	4.57	22.73	73.75	181.81	19.67	16.17	2.30	484.83	5.24
E3-9-2	E3	0.13	19.78	12.27	1.18	188.33	9.33	4.53	28.22	46.56	126.63	25.13	25.50	1.74	337.67	6.70
D6-1-15	E4	0.13	66.68	15.78	1.46	187.11	8.13	3.90	26.95	69.06	192.36	24.78	18.85	1.82	512.97	6.61
C3-2-7	E5	0.23	51.17	14.03	0.84	184.09	7.81	4.18	37.44	66.64	170.47	33.22	23.28	2.74	454.57	8.86
D3-9-4	E6	0.18	40.77	17.41	1.18	177.07	9.01	4.87	28.74	82.25	292.15	42.10	25.77	2.54	779.07	11.23
C5-5-1	E7	0.08	44.35	5.88	1.36	175.67	7.25	3.46	29.37	66.69	562.88	59.42	25.38	2.35	1501.00	15.84
E4-2-1	E8	0.17	64.67	13.97	1.01	182.81	7.95	4.13	34.03	77.80	198.59	34.44	22.05	2.15	529.58	9.18
D5-9-15	E9	0.12	54.48	20.78	1.00	157.79	7.68	3.57	28.34	74.54	238.81	43.19	19.40	2.55	636.83	11.52
D2-12-3	E10	0.27	47.51	16.70	0.89	166.32	6.75	3.51	28.42	74.15	316.32	31.83	20.67	2.22	843.52	8.49
D5-13-1	E11	0.72	90.04	23.30	1.62	178.95	6.82	3.47	22.54	68.69	256.44	42.99	22.07	2.10	683.83	11.47
D2-11-11	E12	0.17	89.43	15.99	1.41	165.65	7.03	3.56	21.22	66.17	342.40	36.02	21.31	2.26	913.07	9.61
C4-1-1	E13	0.16	16.32	9.80	0.81	161.57	8.39	5.23	25.43	91.86	477.25	74.10	29.00	3.01	1272.67	19.76
CHETAK	C1	0.13	19.66	19.66	0.95	184.72	6.23	3.05	19.89	57.87	178.21	13.76	18.31	1.36	475.22	3.67
RVA100	C2	0.09	46.57	13.01	0.90	169.54	6.03	2.82	29.21	58.70	148.42	14.14	18.83	1.59	395.78	3.77
JA20	C3	1.06	50.33	12.94	1.18	179.48	6.89	2.96	21.47	58.68	149.15	13.95	16.97	1.90	397.73	3.72
JA134	C6	0.09	40.61	28.86	1.08	193.97	5.91	3.18	10.61	63.38	203.04	9.69	16.14	1.64	541.45	2.59
POSHITA	C4	0.12	35.66	19.55	0.61	169.26	8.39	5.19	23.04	79.01	193.48	33.91	24.64	2.24	515.93	9.04
NIMITLI118	C5	0.18	40.37	20.35	0.79	171.90	9.67	5.09	26.52	107.97	367.96	63.40	27.35	3.27	981.23	16.91
SE		0.04	2.73	0.72	0.03	1.59	0.26	0.15	1.11	2.09	17.38	2.52	0.59	0.08	46.83	0.60
CD 5%		0.04	15.51	5.57	0.04	12.71	2.95	1.51	10.45	14.56	151.09	18.22	5.86	1.09	102.26	1.112
CV%		4.66	9.85	9.76	1.21	4.35	23.22	23.32	23.80	12.42	38.43	13.11	16.37	15.21	4.66	3.77

Net Plot Size 3x2.7m2; spacing25X15cm
LWLD, RWLD &WA is described in mg/g
SY, RY is described in Kg/plot
Dry herb (DH), Dry root (DR) is described in quintal/ha

Table 3: Description of the proposed Advance breeding line

Trait	CIM-Pushti	Jawahar Asgandh 20
Leaf	Highly wavy, with 7.39 x 3.64 cm dimensions	Non wavy, 6.89 x 2.96cm dimensions
Stem	Strong semi erect culm angle 45-55^0	Spreading, culm angle 65-55^0
Berry	Yellow orange berry berry, diameter 4.37 mm and test weight 124.8mg	Yellow orange berry, diameter 45.22mm and test weight 90.87mg
Root	smooth with even fracture, 21-25cm long starch to fiber ratio of 1.23	smooth with even fracture, 12-18cm long starch to fiber ratio of 1.18
Plant Height	Semi-dwarf (68.59cm)	Dwarf (58.59cm)
Maturity	165-185 DAS	178-190 DAS
Dry herb (q/ha)	629.42	397.73
Dry Root (q/ha)	10.09	3.72
Resistance to leaf blight	Tolerant	Susceptible
Withanolide A (mg/g)	0.785 mg/g dry weight basis	1.01 mg/g dry weight. basis
Withanolide B (mg/g)	0.460 mg/g dry weight basis	0.248 mg/g dry weight. basis
Withanone (mg/g)	0..006 mg/g dry weight basis	0.020 mg/g dry weight. basis

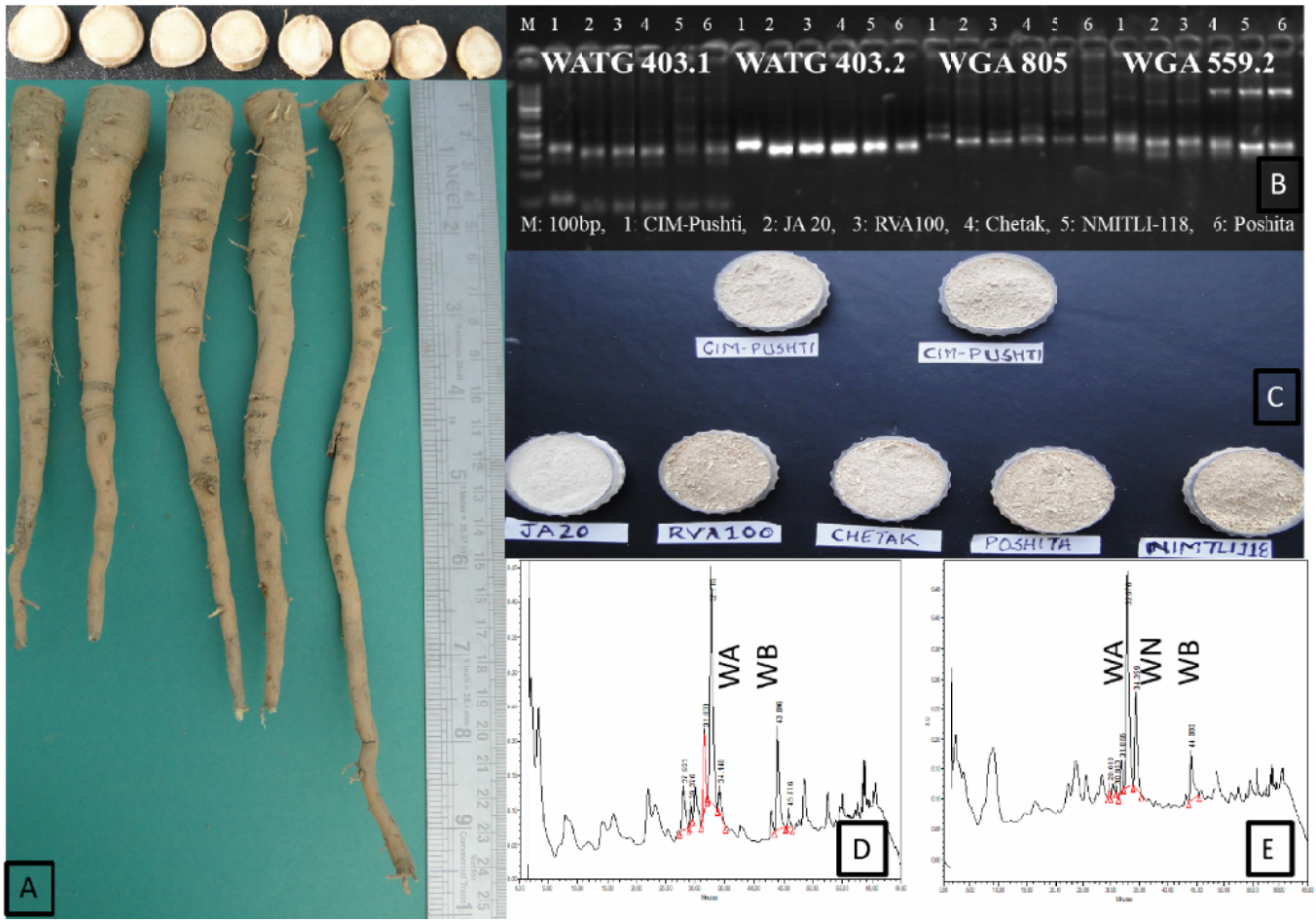

Fig. 2: A: Fresh transverse section and intact roots of CIM Pushti; B: Unique SSR markers distinguishing CIM Pushti (1) from JA20 (2), RVA100 (3), Chetak (4), NIMTLI-118 (5), Poshita (6); C: Pulverised dry root powder indicating starch to fibre ratio; D,E: Root tissue derived RP-HPLC chromatogram for absolute quantification and identification of Withanolides A, Withanone and Withanolide B in CIM Pushti D and in Jawahar Asgand 20 (E)

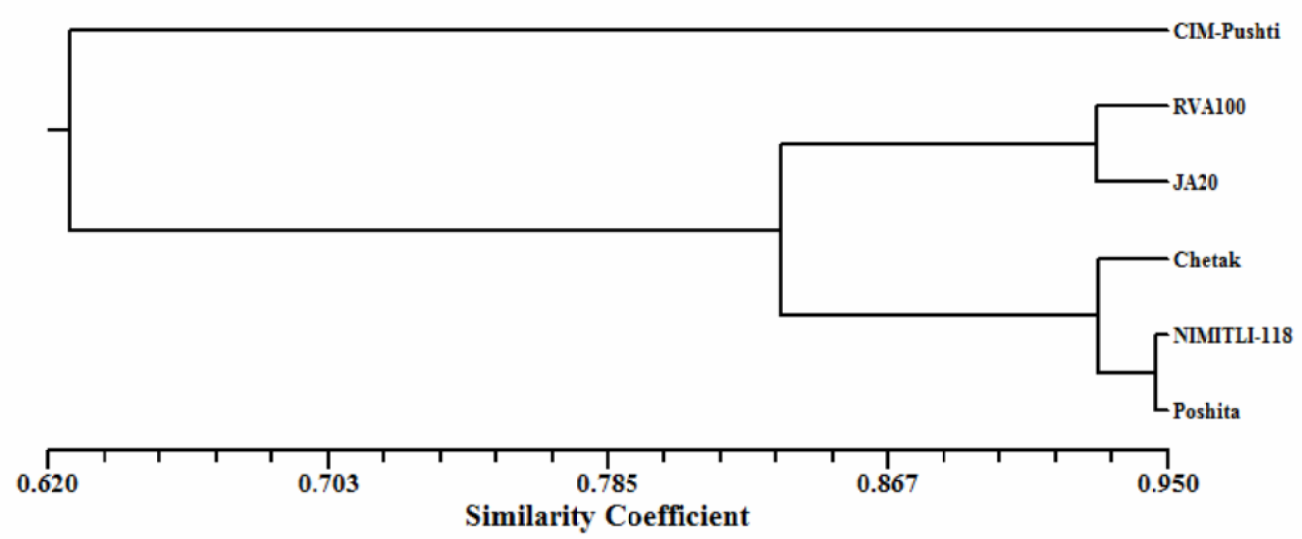

Fig. 3: Dendrogram obtained by UPGMA analysis using SSR markers and DICE similarity Coefficient with r=0.965 representing goodness of the fit of the dendrogram.

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