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MINI-REVIEW

Genetic diversity assessment and biotechnological aspects in *Aristolochia* spp.

Shreya Nath[1] · Nilanjana Ghosh[1] · Tauhid Alam Ansari[1] · Avinash Mundhra[2] · Manoj Tukaram Patil[3] · Abhijit Mane[4] · Abilash Valsala Gopalakrishnan[5] · Md. Habibur Rahman[6] · Manoj Kumar[7] · Radha[8] · Mimosa Ghorai[9] · Subhabrata Paul[1] · Abhijit Dey[9]

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Abstract

Aristolochia, belonging to the family *Aristolochiaceae*, has immense ecological significance due to its large size and huge geographic distribution. In the context of dealing with a genus with a huge number of species like *Aristolochia*, these markers come in handy to precisely identify a particular species and enumerate the genetic diversity. Also, certain species of *Aristolochia* are economically important due to the presence of secondary metabolites and vast use in traditional and modern medicine. But, the presence of profitable biochemical constituents in *Aristolochia* is very low and the breeding process of the plant is highly dependable on pollinators. Hence, identifying different biotechnological approaches to fasten the reproductive cycle of *Aristolochia* and increase the secondary metabolites is of great interest to the researchers. In this study, a comprehensive review has been established on different types of morphological/anatomical markers (starch grains with "Maltese cross"), phytochemical markers (aristolochic acid, triterpenoid, aristolactam etc.) and genetic markers (ISSR, SSR, DNA bar-coding) for various *Aristolochia* spp. We have also discussed the applications of different biotechnological tools in *Aristolochia* spp. which include discrete approaches to promote in vitro germination, in vitro shooting, root induction, somatic embryogenesis, synthetic seed production, acclimatization and hardening and sustainable production of secondary metabolites. In a nutshell, the present review is a first of kind approach to comprehensively demonstrate the genetic diversity studies and biotechnological aspects in *Aristolochia* spp.

Key points

- *Insights into the* in vitro *propagation of Aristolochia spp.*
- *In vitro production and optimization of secondary metabolites.*
- *Assessment of genetic diversity by molecular markers.*

Keywords *Aristolochia* · ISSR · DNA barcoding · Aristolochic acid · In vitro culture · Synthetic seed

Introduction

Aristolochia is a large genus of flowering plants having a large geographical distribution almost all over the world in various diverse climatic situations. The members are often called birthwort, due to the resemblance of the flower with the birth cannel (Ansari et al. 2021; Qin et al. 2021).

Aristolochia is considered to be toxic due to the presence of aristolochic acids (AAs) which causes chronic renal failure, urothelial malignancies and tubulointerstitial fibrosis together termed as aristolochic acid nephropathy (AAN) in both humans and mice (Jelaković et al. 2019 Ji et al. 2021). Consumption of food containing AA through traditional medicine or ingestion of food that is environmentally contaminated with AA can cause progressive renal interstitial fibrosis that frequently leads to AAN. AA intoxication leads to haemodynamic abnormalities by causing an increase in endothelin and reduction in renal nitric oxide levels. Due to such an imbalance in vasoactive factors, vasoconstriction happens to lead to hypoxia, tubular injury, inflammation and ultimately fibrosis.

✉ Subhabrata Paul
subhabrata.dbs@presiuniv.ac.in

✉ Abhijit Dey
abhijit.dbs@presiuniv.ac.in

Extended author information available on the last page of the article

However, *Aristolochia* have widespread use in various regions. Like in Europe, *Aristolochia* sp. were introduced by the Greek scholar Dioscorides (first century), and aristolochic acid and its extracts isolated from *Aristolochia clematitis* were formerly approved to treat against eczemas, abscesses and different long-lasting dermatosis and as a stimulant for immune system (Scarborough and Fernandes 2011; Pohodina et al. 2019). The whole plant of *Aristolochia* is considered to hold different types of medicinal properties in Indian traditional medicine. The roots of *Aristolochia* are designated as anti-inflammatory, diuretic and cardiotonic due to the presence of an alkaloid, aristolochin; leaves are useful for the treatment of cholera and intermittent fever in children; and seeds are traditionally used against inflammation, biliousness and dry cough (Padhy 2021). Hence, a throughout heath risk assessment should be done before using this kind of drugs for any medicinal practices.

On the other hand, various species of *Aristolochia* (viz. *A. longa, A. triangularis, A. bodamae, A. longa* etc.). and their compounds have been reported to demonstrate profound antimicrobial properties (Pereira et al. 2018; Dalcol et al. 2021; El Omari et al. 2020; Ozen et al. 2020; Doudach et al. 2022). Therefore, in vitro cultures can be used as an exciting option to produce and characterize the antimicrobial phyto-constituents from the plant. Besides the genus is also known to host many endophytic microbes (Guevara-Araya et al. 2020, 2022) which can also be cultured in vitro for the production of secondary metabolites. Aristolochic acid (AA) has the potential inhibitory role against snake venom L-amino acid oxidase (LAAO). As AA is notorious for its genotoxic activity, its non-toxic artificial hydroxyl and chloroderivatives can be used for such purpose (Bhattacharjee et al. 2017).Improvements in in vitro techniques offer novel strategies to the viable processing of even threatened and endangered plants and their economically and industrially promising secondary metabolites. In vitro propagation of medico-botanicals and in vitro optimization of their bioactive principles have been done in many plants for the commercial production of therapeutically active plant-based medicines. In plant tissue culture, the production of secondary metabolites has been reported from various species (Jayaprakash et al. 2021; Manokari et al. 2021; Shekhawat et al. 2021; Swamy et al. 2021). Many reports involve the use of different elicitors to increase the quantity of plant secondary metabolites (Dey et al. 2019, 2020; Nandy et al. 2021; Pandey et al. 2021; Nazir et al. 2021a). The present review encompasses an outline on the in vitro propagation and biotechnological tools as an aid for clonal propagation and production of aristolochic acid and allied compounds. In addition, for its use as multipurpose drug in traditional and modern medicines and presence of important toxins, tissue culture *Aristolochia* is a better way out for faster growth, low dependence on natural pollinators and to conserve its short genotype. *Aristolochia* is exploited as a model plant for in vitro regeneration and formation of artificial seed (Remya et al. 2013), clonal propagation (Sarma and Tanti 2017b) and somatic hybridization (Bliss et al. 2009).

A study of markers not only help to distinguish between different population and individual plant itself but also provide insights into the different genetic arrangement, phytochemical constitutes and morphological parameters of one group of plants (Kaur et al. 2019; 2021; Nazir et al. 2021b; Tikendra et al. 2021a,b). Different types of morphological, phytological and genetic markers of *Aristolochia* is well established to differentiate the species of this large genus. Also such markers can be useful as a genetic and breeding tool for crop development (Chesnokov et al. 2020; Desai and Tatke 2019). Therefore, methodologies for measuring genetic diversity and/or proximity in different species utilizing various markers have also been elucidated (Table 1).

Distribution

Aristolochia spp. is a genus present in the family of *Aristolochiaceae* which include approximately 550 species (Cai et al. 2020). All these species are distributed generally under tropic, subtropic, Mediterranean and temperate regions of the world including America, Asia and Africa (Fig. 1). *Aristolochia* subgenus *Siphisia* include 70 species in which 50 of them are found in the southern and eastern parts of Asia, while 20 of them are found in the central and northern parts of America (Do et al. 2015). *Aristolochia ringens* species is found in the tropical regions of America and Africa, whereas *Aristolochia elegans* species are native for South America, while it is exotic for North America, Africa and Asia. Vietnam's tropical, humid and forest region favour the growth of species like *Aristolochia bidoupensis* (van Do et al. 2016). *Aristolochia delavayi*is widely distributed in southwest China along with the Jinsha river valley at an altitude of 1220–2250 m (Yu et al. 2021). In India, 18 species of the genus *Aristolochia* is found, in which *Aristolochia maxima* is found in north Western Ghats (Tilari Ghats) of Maharashtra (Pandurangan and Deepu 2018). This species is native for central and South America. Another species *Aristolochia indica* is found throughout tropical, subtropical and Mediterranean countries including India, Nepal and Bangladesh (Dey and De 2011; Sarma et al. 2018). In Africa, generally on the western side, Madagascar favours the growth of 11 species which include *A. albida*, *A. baetica*, *A. bracteolate*, *A. embergeri*, *A. heppi*, *A. hockii*, *A. fontanesii*, *A. paucinervis*, *A. pistolochia*, *A. rigida* and *A. sempervirens* (de Groot et al. 2006). There are three medicinal and endemic *Aristolochia* species from eastern India

Table 1 Different types of markers found in *Aristolochia* species and populations

Markers	Result/responses	References
Morphological markers	Investigated the root anatomy of *A. indica* by pharmacognostic profiling	Sudhakaran (2014)
	Found separated secondary xylem	
	Found thin-walled cork cells, small cortex and inner cortical cells interspersed with groups of stone cells	
	Found deposited starch in parenchymatous cell of medullary rays	
Phytochemical markers	Analysed the *A. baetica* for the phytochemicals markers like polyphenol, alkaloids, flavonoids, saponins and tannins	Bourhia et al. (2019)
	In *A. bracteolata* alkaloids, glycosides, saponins, starch and protein were analysed. Aristololochic acid and triterpenoid as distinctive phytochemical markers were seen	Avchar (2021)
	Eight aristololactams and 5 aristolochic acid derivatives were reported in *A. moupinensis*, and 6 aristololactams and 3 aristolochic acids were reported in *A. cathcartii*	Zhang et al. (2016)
	Eight aristololactam-type alkaloids and 7 aristolochic acid derivatives were found in *A. tagala*	Liu and Zhang (2020)
	Found 75 essential oil compounds in 10 *Aristolochia* species which classify the species into 4 morphological groups based on principal component analysis	Francisco et al (2008)
	Twenty accessions of *A. indica* were assessed for aristolochic acid content	Dey et al. (2021a, b)
(Molecular markers) ISSR	Evaluation of genetic diversity within 4 *Aristolochia* species *A. indica, A. cathcartii, A. saccate* and *A. tagala* by using 8 ISSR primers	Sarma and Tanti (2017b)
	Check the 9 out of 66 PCR amplicon's bands which are similar in terms of evolutionary history	
	Check the dendrogram of these 4 species for genetic diversity	
SSR	Evaluation of genetic diversity of *A. delavayi* in 193 individuals from ten different natural populations by using 15 pairs of microsatellites SSR primers	Yu et al. (2021)
	Analyse through AMOVA for genetic diversity among the population	
	Analyse the genetic diversity due to restriction of gene flow of *A. delavayi* population along with the Jinsha river	
DNA barcoding	Evaluation of polymorphism within 11 species of *Aristolochia* from Thailand by using *rbc*L, *mat*K, ITS2 and *trn*H-*psb*A as markers	Dechbumroong et al. (2018)

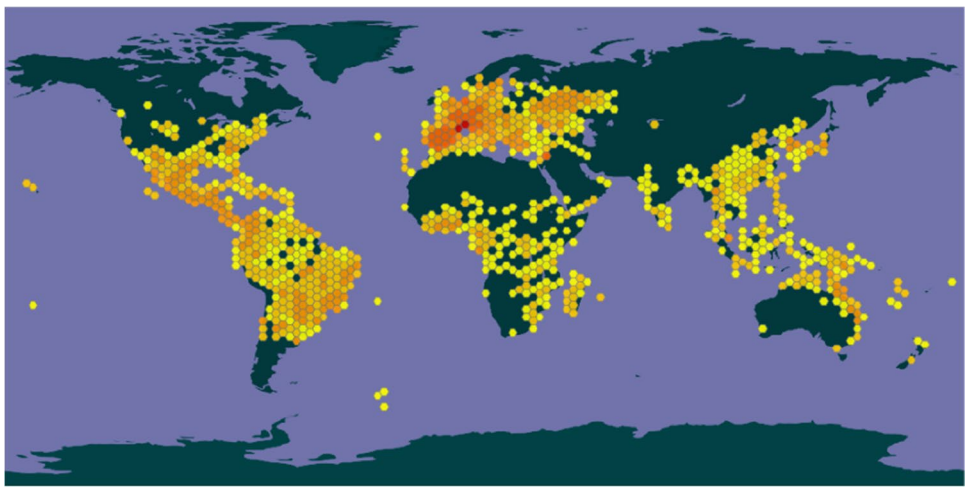

Fig. 1 Worldwide distribution of *Aristolochia* L. (source: https://www.gbif.org/species/2873978)

which have been reported namely *Aristolochia indica* Linn., *Aristolochia saccata* Wall. and *Aristolochia cathcartii* (Sarma and Tanti 2015). *Aristolochia delavayi*, another medicinal and endangered member of Aristolochiaceae, is endemic to China. They generally grow in the warm and dry areas along the Jinsha river (Yu et al., 2021).

Botanical description

Aristolochia is the type of genus of the family *Aristolochiaceae* or the birthwort family having over 450 species in 3 subgenera; *Aristolochia*, *Siphisia* and *Pararistolochia* (Wanke et al. 2006; Yan and Ma 2019). Among them *Aristolochia* itself has approximately 350 species. They are generally evergreen or deciduous lianes. But some deciduous herbaceous members from Mediterranean region are also investigated (Neinhuis et al. 2005). Leaves are found to be 3–7 lobbed, most of the time cordate, 3 (*Aristolochia dalyi*) — 7 nerved (Gonzalez 1998). In *Aristolochia longissima* and *Aristolochia ornithorhyncha*, conspicuous pellucid gland dots are present with the leaves (Jimenez et al. 2021). Flowers are irregular, either solitary or in groups and axillary in position. Perianth is colourful, tubular with various bending, generally flattened at the base but narrow near the throat and pubescent on the inner side; limbs can be either open or recurved, entire or lobbed. Stamens are 6 or more; anthers sessile adnate to the stigma or the back of style. Style is short and lobed near the apex with usually 6-locular inferior ovary. Fruit is capsule, and septicidally dehiscent; seeds are compressed (Britannica, T. Editors of Encyclopaedia, 2013).

In some instances, various authors showed their disagreement with these common morphological features of the different species of such vast genus like *Aristolochia*. Like, González and Stevenson had interpreted the perianth of *Aristolochia*as a trimerous calyx based on morphology, position, development and juxtaposition with the closely related taxa (González and Stevenson 2000) after studying 42 species.

Adams et al. have done a comparative study between 4 closely related species of *Aristolochia* from all over the world namely *A. californica*, endemic to California, *A. macrophylla* and *A. tomentosa* of eastern USA and *A. manshuriens* of eastern Asia based on seed characters (like seed mass, surface area wings, state and shape of embryo). It was found that though having similar morphological characteristics of the embryo (linear and underdeveloped), those features are statically different which assign them into 4 separate species (Adams et al. 2005).

Aristolochia was found to have a profound trapping mechanism for pollination in case of in proterogynous flowers. Experiments on 6 Mediterranean species have shown during female flowering stage an array of well-developed trichomes present on the underlying surface of the flower which ensure the entry of the pollinators into the flower but inhibit their exit. But after the flowers enter the stages of anthesis, the inner surface characters of the flower modify to enable the insect to escape (Oelschlägel et al. 2009).

Genetic diversity assessment

Morphological markers

Morphological markers are visual phenotypic characters of a plant that is particular to the certain species or genus. But the main disadvantage of these markers is that they are few in number and always influenced by environmental factors and covers of the entire genome (Chesnokov et al. 2020).

Sudhakaran has investigated the root anatomy of *Aristolochia indica* through pharmacognostic profiling and found the transverse section having a circular outline with tissue organization of thin-walled cork cells, small cortex and inner cortical cells interspersed with groups of stone cells. Secondary xylem was separated to form narrow strips. Wide medullary rays had greater quantities of parenchyma with ray cells having deposition of starch. Solitary vessels are occluded with tyloses and starch grains with 'Maltese cross'. He suggested these specific anatomical characters might be an authentic marker of this taxon (Sudhakaran 2014).

Phytochemical markers

Phytochemical markers are chemical constituents made up of single or group of herbal drugs with a well-defined chemical structure. These constituents may or may not possess any medicinal characteristics but are useful for quality control of plant-based drug or formulation (Desai and Tatke 2019). The genus contains a number of constituents which can be utilized as important phytochemical markers (Fig. 2).

Aristolochia baetica is a wild member of *Aristolochia* from Morocco whose roots have been used against cancer from a long time ago. The root aqueous extract of *A. baetica* was subjected to preliminary qualitative phytochemical screening to find out about the phyto-organic component behind their biological activities. The presence of polyphenol, alkaloids, flavonoids, saponins and tannins has been observed (Bourhia et al. 2019). Similar study also was done on the methanolic root extract of *A. bracteolata* which has also shown the presence of alkaloids, glycosides, saponins, starch and protein. High performance thin-layer chromatography fingerprinting study revealed the occurrence of aristololochic acid and triterpenoid as distinctive phytochemical markers of the particular plant (Avchar er al., 2021).

In a study to explore the phytochemical constituents of *Aristolochia moupinensis* and *Aristolochia cathcartii*, 8 aristolactams and 5 aristolochic acid derivatives were

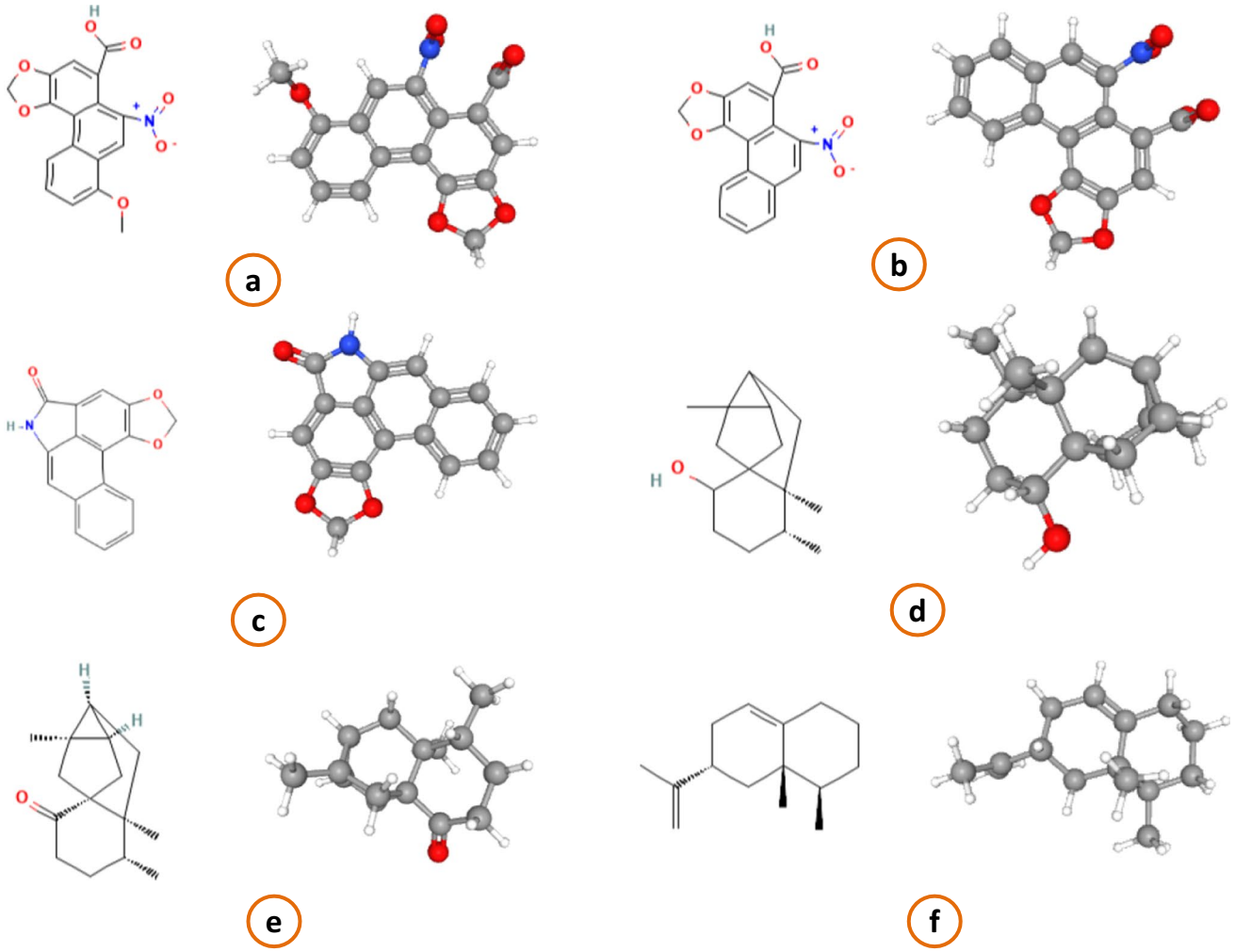

Fig. 2 2D and 3D chemical structures of the bioactive compounds of *Aristolochia* spp.: **a** Aristolochic acid I, **b** Aristolochic acid II, **c** Aristololactam II, **d** Ishwarol, **e** Ishwarone, **f** Aristolochene (source: www.PubChem.com)

isolated from *A. moupinensis*, whereas 6 aristolactams and 3 aristolochic acids were isolated from *A. cathcartii* (both were isolated from whole herb). Aristolactam I, aristolactam AII, aristolochic acid A and aristolochic acid BII were found from both of the species. Aristolactam-type alkaloids and aristolochic acid derivatives were widely spread in all the plant members of genus *Aristolochia*. Aristololactam is an intermediate in the biosynthetic pathway for aristolochic acid. The aristolochic acids in the *Aristolochia* possessed a unique chemical structure and are responsible for the same biological property of *Aristolochia* species. Hence, aristololactam-type alkaloids and aristolochic acids derivatives might be hypothesized to be unique for the particular genus, thus could be used as potential phytochemical markers for the genus (Zhang et al. 2016).

Eight aristololactam-type alkaloids and seven aristolochic acid derivatives were isolated from the whole plant of *A. tagala*. Among them sauristolactam and 7-methoxyaristololactam IV are very unique to the species. Sauristolactam is not found in any other *Aristolochia* species and though the occurrence of 7-methoxyaristololactam was observed in some of the Asian members of *Aristolochia*, it is quite rare. Hence, both of them could be used as chemotaxonomic or phytochemical marker of the species (Liu and Zhang 2020).

After analysing the essential oils from the roots of 10 *Aristolochia* species by GC–MS, 75 compounds were found. Multivariate analysis of those chemicals from roots classifies the 10 species into 4 morphological groups based on principal component analysis. The groups were identified by principal component 1 (monoterpenes, like a-thujene) and principal component 3 (sesquiterpenes, such as germacrene A (52), c-elemene (39) and b-gurjunene) (Francisco et al. 2008).

Group 1: A. arcuata, A. chamissonis, A. lagesiana, A. melastoma and A. pubescens.
Group 2: A. gigantean (highest positive PC1).
Group 3: A. elegans (highest positive PC3).
Group 4: A. esperanzae, A. galeata, and A. malmeana.

Molecular markers

ISSR

Inter-simple sequence repeats (ISSR) is a molecular technique which helps in the determination of multi-locus marker (repetitive sequence present in genome of organism) by the help of PCR by using microsatellite sequence as primers. The amplicons produced by this technique help in the study of evolutionary history, genetic diversity and gene mapping of closely related species. Forty-five Passion fruit (*Passiflora* sp.) accession was selected to check against eighteen ISSR primers. The result obtained from the mean Shannon–Weaver diversity index was 0.32 which represents a good diversity in the selected *Passiflora* germplasm by the ISSR markers (Santos et al. 2011). In another study, 23 mango germplasm accessions were collected from Guangxi province, China and checked against 18 ISSR primers. The result showed out of 156 bands, 87 are polymorphic. It can be concluded among the other cultivars, the genetic similarities in Xiang Ya mango type and their progenies are very high (Luo et al. 2011).

To determine the genetic variation of *Aristolochia s*pecies, 8 ISSR primers are used for the 4 species from Assam. These species are *A. indica*, *A. cathcartii*, *A. saccata* and *A. tagala*. It is found that *A. indica* and *A. cathcartii* (Cluster-I) are 62% similar in terms of genetic variation while *A. saccate* (Cluster-II) is 28% similar with Cluster-I species, while *A. tagala* (Cluster-III) is 22% similar with *A. saccate*. Nine out of 66 PCR amplicon's bands of these four species are similar indicating that they are sharing common evolutionary history. Remaining bands are the proof for the genetic divergence present in the species and showing polymorphism in their character. Dendrogram of these 4 species also shows the same genetic similarity for *A. indica* and *A. cathcartii*, while the other two species are not much closely related due to divergence in the genes (Sarma and Tanti 2017a).

SSR

Simple sequence repeat is a microsatellite DNA repeat found in genome having a high rate of polymorphism and widely distributed within the eukaryotic organisms, and the number of repeat is very specific and varies between organisms and helps in the assessment of genetic diversity between closely related organisms having minimal characteristic differences. Twenty-nine cucumbers (*Cucumis sativus* L.) accessions were selected to estimate the genetic diversity by comparing 13 genomic microsatellites (gSSR) and 16 expressed sequence tag (EST)-SSR (eSSR) markers. The dendrogram produced individually from the results of both of these markers shows similarity in the position of most of the cucumber germplasm. Comparing the data from eSSR markers, independent sub-clusters can be identified containing five germplasms. They all are resistant to downy mildew concluding a probable connection between those eSSR markers and disease resistance of plants (Hu et al. 2011).

SSR analysis is performed for the analysis of genetic diversity and difference in genetic makeup of the *Aristolochia delavayi* species with respect to its wild type. Fifteen pairs of microsatellite SSR primers are used for 193 individuals from ten natural populations. Through AMOVA (analysis of molecular variance), 68.4% genetic diversity is seen within the population, whereas 31.6% genetic diversity is seen among the population of *Aristolochia delavayi*. High genetic diversity within the population is due to outcrossing within the population by sexual reproduction or due to the retention of genetic resources. Restriction of gene flow may cause the less genetic diversity among the population which restricts the reproductive abilities of plants.

Since *Aristolochia delavayi* is found along the Jinsha River and warm and dry areas of China, so this kind of geographical habitat restricts the exchange of gene among the species. Another reason is that pollination of this species mainly depends on the family Ceratopogonidae and Chironomidae pollinator which are less efficient and have weak flying abilities and warm and dry climate does not favour the dispersal of seed due to which gene flow is restricted within the species and cause major genetic diversity within the population (Yu et al. 2021).

DNA barcoding DNA barcoding is the technique in which short sequence of DNA or organelle DNA is used to identify and help in the comparison of genetic diversities among species. The genes which are used as DNA barcode include cytochrome *c* oxidase I (COI or COX1) coding gene present within mitochondrial DNA, internal transcribed spacer (ITS) rRNA etc. due to their less variation in intraspecific level compared to interspecific level in species. Four plastid coding genes (rpoB, rpoC1, rbcL and matK) and 3 noncoding spacers (atpF-atpH, psbK-psbI and trnH-psbA) based on the chloroplast genome sequence of *Lemna minor* as proposed by the CBOL (Consortium for the Barcode of Life) were used to distinguish 97 accessions representing 31 species of Lemnaceae (aquatic monocots). It can be concluded that among other genes chosen in the study, the atpF-atpH noncoding spacer could be used as a universal DNA barcoding marker for species-level identification for Lemmaceae based on reliable amplification, straightforward sequence

alignment and rates of DNA variation between species and within species (Wang et al. 2010).

Recent studies on DNA barcoding use *rbc*L, *mat*K, ITS2 and *trn*H-*psb*A as markers to identify the genetic variability and polymorphism within the 11 species of *Aristolochia* from Thailand. This evaluates the highest variations which are found in ITS2 (28.98%) region of DNA followed by *rn*H-*psb*A (11.56%), *mat*K (11.15%) and *rbc*L (3.29%) in all 11 species of *Aristolochia* (Dechbumroong et al. 2018).

Achievements made in *Aristolochia* through modern biotechnological tools

In vitro germination

In vitro germination is an easier method to propagate for conservation and economic purposes (Table 2). The in vitro germination of *Cannabis sativa* seeds has faced some difficulties regarding uniformity and germination time due to issues in the standardization of disinfection procedures. A recent study has been mediated by using the generalized regression neural network (GRNN) to assess the type and concentration of disinfectants and the time of immersion for in vitro germination of the seeds. The results showed that treating the seeds with 4.6% sodium hypochlorite along with 0.008% hydrogen peroxide for 16.81 min manifested the best results with a 0% contamination rate and 100% germination after scarification within 1 week (Pepe et al. 2021).

The presence of scanty endosperm and linear, small embryo of *Aristolochia tagala* makes the germination of the seeds under normal conditions inconvenient (Biswas et al. 2007). Therefore, these factors have driven the utilization of different techniques of in vitro germination in order to enhance the viability of seeds and propagation rate. A report by Krishnan et al. (2019) suggested that exposure of seeds presoaked in warm water (50 °C) for 5 min to a germinator not only amplified the germination percentage but also successfully reduced the number of days taken for germination. The percentage of germination, however, was found to be greater in contrast to presoaked seeds exposed to an open room. According to Bhat et al. (2020), presoaking of seeds with warm water tends to stimulate the softening of seed coat and promotes rapid protrusion of tip of radicle upon attaining maturity. GA_3, KNO_3 and thiourea treatment at varying concentrations was found to have a pronounced effect on the rate of germination and effectively reducing the time taken for germination. Thiourea, a potential agent of in vitro germination, elevates endogenous cytokinin levels to alleviate inhibition on seed coat.

In vitro shooting

Morphogenesis from callus culture using a wide array of explants including adventitious shoot, nodes, internodes and leaves is beneficial for developing multiple clones of endangered medicinal plants. In vitro organogenesis can be manipulated by regulating the concentration of plant growth regulators (PGRs) specifically auxin and cytokinin. Though cytokinin along with auxin has a positive impact on shoot proliferation from callus, higher concentration of BAP (benzyl amino purine) and KIN (kinetin) can counteract this activity. It is also essential to utilize phloroglucinol to support in vitro shooting since accumulation of phenolic compounds may interfere with the normal phenomena and turn the callus black. In a study conducted on rice to standardize a reproducible and highly efficient plant regeneration protocol, the highest shoot regeneration was observed on the Murashige and Skoog (MS) medium supplemented with 2.0 mg/L benzylaminopurine, 0.5 mg/L 1-naphthaleneacetic acid, 500 mg/L proline and 500 mg/L glutamine in the callus obtained from MS medium complemented with 2.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 500 mg/L proline and 500 mg/L glutamine (Pawar et al. 2015).

The most effective result of shoot organogenesis in *Aristolochia* from callus culture was recorded with BAP in combination with NAA. Media of 2 µM BAP, 1 µM NAA (1-naphthalene acetic acid) and 10 µM PG in MS (Murashige and Skoog) or MS media fortified with 2,4-D (2,4-dichlorophenoxyacetic acid) and KIN provided prominent results (Remya et al. 2013). The capability of NAA to initiate shoot organogenesis was put forward by Biswas et al. (2007), where 79% explants cultured showed successful shooting with 2 mg/L BAP and 0.5 mg/L NAA in *A. tagala*. In *A. saccarta* and *A. cathcartii,* 3–4 mg/L BAP and 0.5–1 mg/L NAA demonstrated the highest mean shoot length of 4.02–4.34 and the number of shoot in the range of 3.4–6.2 (Sarma and Tanti 2017a, b). Remya et al. (2016) suggested for elongation of shoot from apical bud explant, it is convenient to supplement MS media with GA_3. This report validated that BAP with KIN is even a fruitful alternative for in vitro shoot formation along with 0.1% charcoal fortification. BAP and spermidine, a polyamine, synergistically assisted the proliferation of shoot from nodal explant-derived calli (Dey et al. 2020).

Root induction

Root organogenesis from a wide variety of explants under precisely suitable culture conditions are dependent upon the type and concentration of auxin present in the medium. Germplasm conservation and reduplication *Artemisia* sp. for research and therapeutic purposes through biotechnological interventions have been greatly studied for the past decade.

Table 2 Different types of in vitro culture achieved in *Aristolochia* via tissue culture

In vitro culture type	Plant	Result	Reference
In vitro germination	Seeds of *A. tagala*	Exposure of seeds presoaked in warm water (50 °C) for 5 min amplified the germination percentage and reduced the germination time	Krishnan et al. (2019)
	Seeds of *A. tagala*	Presoaking the seeds with warm water stimulate the softening of seed coat and promotes rapid protrusion of tip of radicle during maturity	Bhat et al. (2020)
In vitro shooting	*A. tagala* (leaf-derived callus)	MS media fortified with 2 µM BAP, 0.5 µM NAA, 10 µM PG promotes shoot organogenesis. Multiple shoots also can be regenerated from encapsulated nodes grown on MS medium supplemented with 3° µM BAP and 0.5 µM kinetin	Remya et al. (2013)
	A. tagala (nodal segment as explants)	Explants (79%) cultured showed shooting after culturing in MS 2 mg/m BAP and 0.5 mg/L NAA	Biswas et al. (2007)
	A. saccarta and *A. cathcartii* (nodal explant)	Application of BAP in combination with NAA showed better shoot induction in comparison to those hormones used separately	Sarma and Tanti (2017a)
	A. tagala (apical bud explant)	Addition of activated charcoal in culture media (MS media) help in circumventing the problem of polyphenol release of explant which hampered the regeneration of adventitious shoots	Remya et al. (2016)
	A. indica (nodal explants)	SH (Schenk and Hidebrandt) media fortified with 2.0 mg/L BAP and 0.5 mM spd (spermidine) showed best results with an average number of 47.5 base callus-derived shoots	Dey et al. (2020)
	A. indica (shoot tip, nodal segment, leaf disc callus, leaf segment)	Murashige and Skoog's medium with 0.54 µM α-naphthaleneacetic acid/NAA and 13.31 µM benzyladenine/BA promoted the regeneration of the maximum number of shoots (45–50) from nodal segment and shoot tip. The best results were observed in terms of shoot bud regeneration from leaf-derived callus while cultured on basal medium fortified with 2.69 µM NAA, 13.31 µM BA and 1.0 mg/l PG. Direct shoots organogenesis from leaf segments was done by using 13.31 µM BA along with 50 mg/L activated charcoal in MS basal medium	Manjula et al. (1997)

Table 2 (continued)

In vitro culture type	Plant	Result	Reference
In vitro root induction	*A. saccarta* (nodal explants)	IBA exhibited surpassing effect on number of rootlets and mean height of roots than NAA in *A. saccarta*	Sarma and Tanti (2017a)
	A. tagala (apical bud explant)	Regenerated shoots are rooted after culturing on MS medium with indole acetic acid/ IAA (1.5 µM), kinetin/KIN (1.5 µM) and 6-benzylaminopurine/BAP (0.5 µM)	Remya et al. (2016)
	A. indica (shoot tip, nodal segment, leaf disc callus, leaf segment)	Rooting the microshoots in White's medium supplemented with 2.46 µM IBA showed 85% survival rate	Manjula et al. (1997)
	A. indica (leaf-derived and nodal calli)	Rooting (80%) along with greater root length was observed in shoots regenerated from leaf-derived calli, while shoots from nodal calli displayed 95% rooting after culturing on MS media supplemented with 0.8 mg/L NAA	Pattar and Jayaraj (2012)
	A. indica (nodal explants)	Spermidine (0.5 µM) along with 1 mg/L IAA in MS media scaled up the rooting and lateral root development	Dey et al. (2020)
Somatic embryogenesis	*A. saccarta* and *A. cathcartii* (nodal explant)	Direct somatic embryogenesis was achieved after culturing the nodal explant on MS medium with different concentrations of BAP (1.0–4.0 mg/L) and 2iP (1.0–4.0 mg/L) separately or in combination with low concentration (0.5 and 1.0 mg/L) of auxin (NAA) respectively	Sarma and Tanti (2017a)
Synthetic seed production	*A. tagala* (nodal explants)	Artificial seeds were formed by encapsulating the nodes in 3% (m/v) sodium alginate and 1% (m/v) $CaCl_2$	Remya et al. (2013)
Acclimatization and hardening of plants	*A. bracteolata*	Survival rate (95%) of in vitro plants can be achieved when acclimatized to soil, farmyard, garden soil manure mixture in the ratio 1:1:1	Sebastinraj and Sidique, (2011)
	A. tagala	Survival rate (80%) rate of in vitro plants can be achieved when acclimatized to vermicute, soil mixture in the ratio 1:1	Remya et al. (2016)
	A. indica	Survival rate (95%) was observed when in vitro plantlets were acclimatized and hardened with White media and vermicute and transferred to greenhouse conditions	Manjula et al. (1997)
	A. tagala	Survival rate (80%) was noted when in vitro plantlets were hardened in plastic pots with soil and manure mixture in 1:1 ratio	Biswas et al. (2007)
	A. indica	Survival rate (100%) of in vitro plants were recorded after hardening the plantlets with autoclaved soil, sand, soilrite acclimatizing them by transferring to large pots with soil and soilrite ratio in ratio of 2:1	Dey et al. (2020)

Recently, to establish a protocol for micropropagation of *Artemisia annua* L, the optimum rooting was observed on the Murashige and Skoog (MS) medium supplemented with 0.1 mg/L IBA in leaf-derived calluses (Zayova et al. 2020).

According to Sebastinraj and Siddique (2011), Sarma and Tanti (2017a, b) and Biswas et al. (2007), IBA (indole-3-butyric acid) is most appropriate for in vitro rooting of *Aristolochia* in contrast to NAA and IAA (indoleacetic acid). On one hand, Sarma and Tanti (2017a, b) reported 0.5 mg/L IBA exhibited surpassing effect on a number of rootlets and mean height of roots than NAA in *A. saccarta*. Remya et al. (2016), on the other hand, observed efficient in vitro

rooting from apical bud explants of *A. tagala* on exposure to an amalgamation of IAA, BAP and KIN. Manjula et al. (1997) stated MS media and low ionic strength White media supplemented with precise concentration of IBA is effectual enough to initiate rhizogenesis and lateral root formation. In vitro rooting potency is also dependent upon the type of explant used. It was recorded by Pattar and Jayaraj (2012) that 80% rooting and greater root length were available in shoots from leaf-derived calli, but shoots from nodal calli displayed 95% rooting. In both cases, rooting was evident when MS media were supplemented with 0.8 mg/L NAA. The effect of other secondary metabolites on the in vitro rooting was put forward by Dey et al. (2020). They proved the fact how polyamine like spermidine (0.5 mM) along with IAA (1 mg/L) in MS media scaled up the rooting and lateral root development.

Somatic embryogenesis

This technology for in vitro propagation of endangered plants has been of immense focus on account of low propagation rate by seeds. The potential of somatic embryogenesis to regenerate plantlets is considered to be far more efficient than adventitious shoot, leaves and apical bud organogenesis. The in vitro propagation of elite palms through somatic embryogenesis is found to be advantageous for the high degree of variability, which will exist among improved progenies. By using such technologies, oil production from oil palm can be increased up to many degrees (Soh et al. 2011). For this context, somatic embryos of *Elaeis guineensis* Jacq (oil palm) were obtained after culturing the thin cell layer sections from the base of the explants in Murashige and Skoog (MS) medium supplemented with 450 µM picloram and 2,4-dichlorophenoxyacetic acid (2,4-D) with 3.0% sucrose, 500 mg /L glutamine, and 0.3 g/L activated charcoal and gelled with 2.5 g/L Phytagel for 12 weeks (Scherwinski-Pereira et al. 2010.

Very few reports have till now suggested methods to develop somatic embryo directly or in directly. Successful somatic embryo formation can be carried out by nodal explants cultured in different concentrations of 2-isopentenyl-adenine (2-iP) and benzylaminopurine (BAP) in combination with naphthaleneacetic acid (NAA) which permitted callus induction. *A. saccata* and *A. cathcartii* low concentrations of NAA positively influenced somatic embryo development, and there has been 88.3–96.6% success rate of regeneration of shoot from explants (Sarma and Tanti 2017b). *A. indicus*-directed callus induction up to 90% in 1:2 of NAA and BAP, respectively (Siddique et al. 2006). It was also observed in some cases cytokinin (Kinetin, BAP) and auxin (NAA, IAA) can be used in 1:2 ratio.

Synthetic seed production

Artificial seeds usually harbour the somatic embryo or explants from mother plant and are an excellent alternative to zygotic embryos as well as conventional plant breeding techniques. Plant species which have a drawback in terms of seed viability, inconvenience faced in vegetative propagation and storage can successfully propagate via synthetic seeds (Rihan et al. 2017). They help in the formation of multiple clones of the target plant thus preserving their genetical identity. To develop an efficient protocol for the production of the synthetic seeds of *Rhinacanthus nasutus* for their faster multiplication and isolation of Rhinacanthin-C, Rhinacanthin-D and Rhinacanthin-N, young healthy cotyledon explants were grown on MS medium supplemented with 4 mg/L 2, 4-D and 0.5 mg/L IBA to develop an embryonic callus. Those calluses are further cultured for 45 days in half-strength MS medium supplemented with 4.0 mg/L indole-3-butyric acid (IBA) to produce several somatic embryos. Somatic embryos at the torpedo stage were suspended in a matrix of MS medium supplemented with sodium alginate (3% W/V), and then dropped into the 100 mM calcium chloride ($CaCl_2{\cdot}2H_2O$) solution to generate the synthetic seeds. The optimum growth ability of the synthetic seed was evaluated on MS medium with 0.2 mg/L gibberellic acid (GA3) (Cheruvathur et al. 2013).

Remya et al. (2013) proposed a protocol for developing artificial seeds in commercially important plant *A. tagala*. In vitro shoot and nodal explants cultured in MS media were allowed to form the bead like seeds using different concentrations of sodium alginate and 1% $CaCl_2$. Most appropriate results were obtained at an intermediate concentration of sodium alginate, and maximum shoot formation was observed when these synthetic seeds were made to propagate in MS media supplemented with 3 µM BAP and 0.5 µM KIN.

Acclimatization and hardening of plants

Acclimatization and hardening are indispensable in order to ensure high chances of survival of in vitro plantlets. Direct transfer of the in vitro plants to the field can be detrimental. In order to overcome this drawback, certain approaches have been made. Sarma and Tanti (2017a, b) reported in vitro grown plantlets could be hardened by 1% w/v bavistine and irrigation with 0.5 × MS inorganic salts consecutively for 7 days. Acclimatization of plantlets was attained by exposing plantlets to aseptic culture room under controlled photoperiod and temperature for 2 weeks. Furthermore, the plantlets were exposed gradually to sunlight for acclimatization and were maintained in a garden. In *A. bracteolata*, 95% survival rate of in vitro plants were confirmed when acclimatized to soil, farmyard and garden soil manure in the ratio of 1:1:1 (Sebastinraj and Sidique 2011). Survival rate of 80% has been reported in *A. tagala* by Remya et al. (2016) when plantlets were acclimatized with vermicute and soil mixture (1:1). *A. indica* was seen to exhibit approx. 95% survival rate

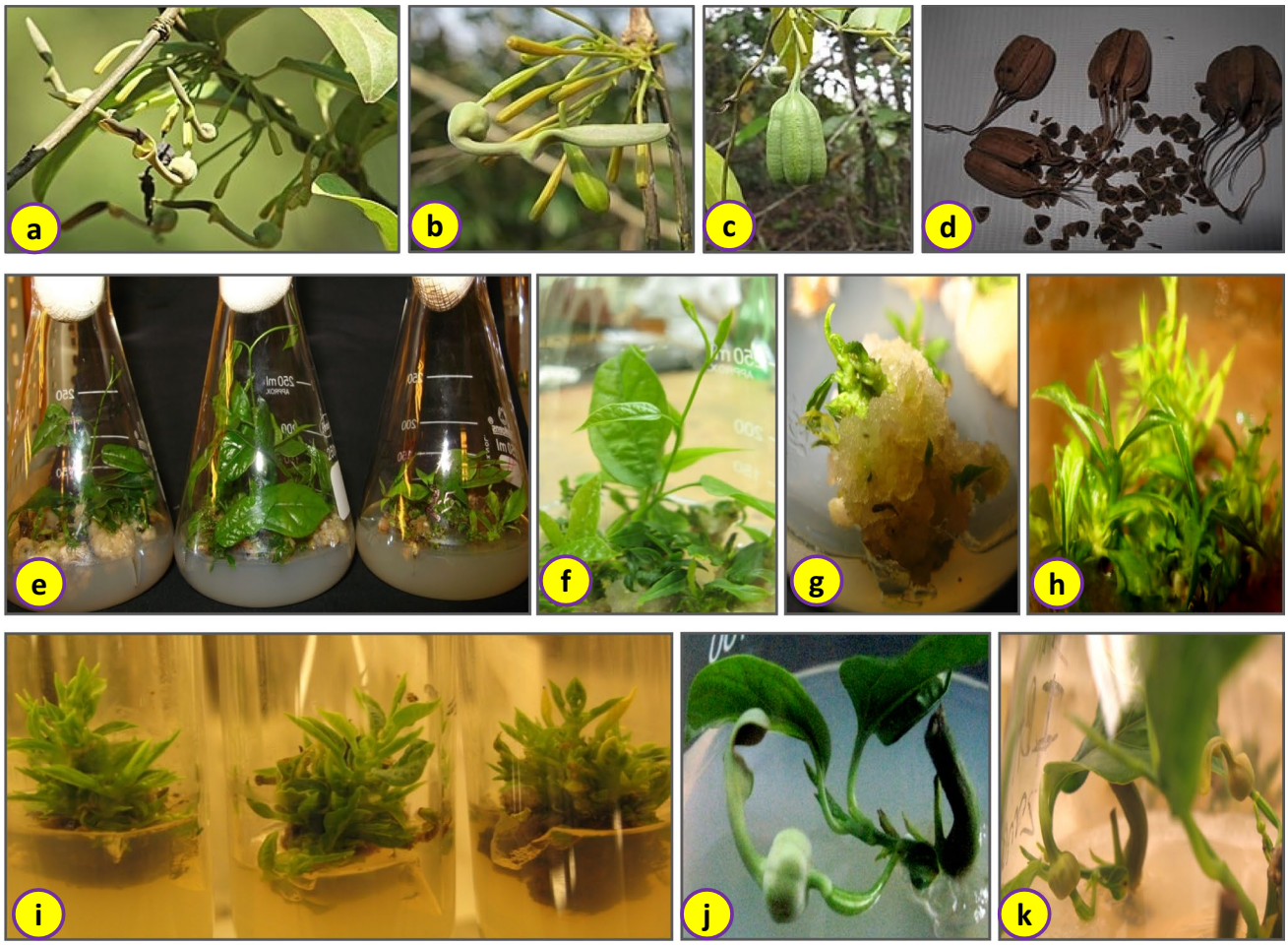

Fig. 3 *A. indica* **a** Habit, **b** Flowers, **c** Fruits, **d** Seeds (source: Wikimedia commons; Creative Commons Attribution-Share Alike 4.0 International); **e**, **f**, **g**, **h**, **i**. In vitro shoot multiplication and in vitro indirect regeneration from callus; **j**, **k** In vitro flowering (**e**–**k**: Abhijit Dey's own research photos)

when in vitro plantlets were hardened with White media and vermicute and transferred to greenhouse conditions (Manjula et al. 1997). Biswas et al (2007) also confirmed 80% survival rate when in vitro plantlets were hardened and acclimatized in plastic pots with 1:1 soil and manure. Survival rate of 100% of in vitro plants were noted when they were hardened with autoclaved soil, sand, soilrite and acclimatized by transferring to large pots, where soil and soilrite were in the ratio of 2:1 (Dey et al. 2020). Figure 3 presents various in vitro tools and biotechnological aspects for plant propagation, regeneration and flowering.

Biosynthesis and regulation of secondary metabolites

Aristolochia sp. is considered to be an essential plant owing to the wide range of pharmaceutically active secondary metabolites available in different parts of the plant. Terpenoids, steroids and phenolic compounds like lignans, coumarins; alkaloids like berberine, aristolochic acid, aristolactams, isoquinolines and benzylisoquinoline are major categories of components extracted from roots, leaves and stem (Kuo et al. 2012). β-Sitosterol and stigmasterol were two major steroids extracted from different plant parts of *Aristolochia* sp. Terpenoids are beneficial for its anti-inflammatory, antibacterial, antiviral and antirheumatic effects and have neutralizing potency against haemorrhagic effect (Dey and De 2011). Aristolochic acid has antisnake venom properties, but recently some reports have highlighted carcinogenic and nephrotoxic effects (Dey et al. 2020).

Biosynthesis of terpenoids

Comprehensive study of the terpenoid content of *Aristolochia* sp. has demonstrated the prevalence of sesquiterpenoids, monoterpenoids and deterpenoids from the plant extracts. Among these, sesquiterpene hydrocarbons like ishwarane, ishwarone, aristolochene, ishwarol, aristolactone, cadinanes,

aristolanes, beta caryophyllene, germacranes and bicyclogermacranes are present in considerable amounts. Pacheco et al. (2009) verified the presence of diterpenoid and its derivative compounds from *Aristolochia* sp. by isolating abundant kaurene, cledorane, labdane and assigned structures by ^{13}C-NMR. Aristolin, another terpenoid isolated, was found to be an ester of aristolochic acid and a diterpenoident-kauran-16-β, 17-diol (Kuo et al. 2012). Preistap et al. (2002) confirmed sesquiterpenes were more abundant, as compared to monoterpenes in leaves and stems. Ishwarone, the tricyclic precursor molecule of ishwarane, undergoes retrosynthetic removal of C8 resulting in the formation of octalone. Methylation of octalone resulted in the formation of decalone which in turn synthesized ishwarane via tertiary alcohols and octalin.

Biosynthesis of alkaloids

Since aristolochic acids are usually derived from benzylisoquinolinealakoids (BIA), having clear knowledge of their biosynthetic pathway is of utmost importance, as this data would be indispensable for having clarity regarding biosynthetic pathway of aristolochic acids. Cui et al. (2022) elucidated the BIA synthesis pathway using *Aristolochia contorta* as experimental model by genome-wide analysis and transcriptomic analysis. Tyrosine derivatives act as a precursor molecule in the pathway along with dopamine and 4 hydroxyphenylacetaldehyde which undergoes condensation in the presence of enzymes coded by NCS 70, 71 to form(S)-norcoclaurine. This product is catalysed by enzymatic activity of 6 OMT, CNMT, NMCH 4′OMT 67, 72–76 to yield s-reticuline as intermediate product. S-reticulline ultimately is responsible for synthesis of alkaloids like berberine.

Sustainable production of secondary metabolites by in vitro techniques

Owing to high demand of pharmaceutically active secondary metabolites in the drug industry, sustainable in vitro production of plants is becoming a necessity. One such instance is enhancing naturally low-calorie sweeteners (Steviol glycosides) obtained from *Stevia rebaudiana*. After culturing the 3-week-old in vitro plantlets on liquid woody plant medium (WPM) supplemented with 100 µM methyl jasmonate (MeJA) for 2 weeks, a 17-fold increase in stevioside production has been observed in comparison to the control plant (plantlet is grown without any elicitors) (Bayraktar et al. 2018). Also, hairy roots of *Nicotiana tabacum* L. cv. Petit Havana SR1 do not produce geraniol naturally. But after the genetic manipulation to express a plastid-targeted geraniol synthase gene isolated from *Valeriana officinalis* L. (VoGES) mediated by *Agrobacterium rhizogenes*, it was observed that the hairy roots can produce geraniol ranging from 13.7 to 31.3 µg/g dry weight (Ritala et al. 2014).

Massive exploitation of this perennial herb has eventually led to its classification as a critically endangered species. This approach is an effective conservational technique which is the sole solution to the exuberant harvesting of *Aristolochia* sp. from Western Ghats and Assam where it is endemic to. As of now, there are limited reports with respect to in vitro production on account of less comprehensible findings regarding the metabolic pathways involved. Certain works have also supported the fact that growth culture conditions and presence or absence of phytohormones precisely regulated the synthesis of secondary metabolites from explants.

Remya et al. (2016) reported the extraction of ishwarane, a tetracyclic sesquiterpene from in vitro plant leaves developed from apical bud explants. This particular method of in vitro regeneration of plantlets also confirmed the isolation of phenols, flavonoids, terpenoids and fatty acids in a sustainable quantity. Leaf-derived callus failed to report the absence of ishwarane but confirmed the presence of certain bioactive compounds unique to this in vitro approach. Alkaloid berberine was successfully isolated by Remya et al. (2016) by *both* in vitro techniques.

It is evident from many reports how culture media enriched with plant growth regulators, secondary metabolites can implement in vitro shooting, rooting, callus induction and multiplication. They are capable enough to simultaneously upgrade the synthesis of bioactive metabolites in in vitro plants. Dey et al. (2020) validated in vitro regeneration of *A. indica* to isolate aristolochic acid and analysed its endogenous level in in vitro shoots and roots. In this species of *Aristolochia* sp., nodal explant and apical shoot buds were cultured in SH media and were enriched with varying concentrations of polyamines (0.5 mM–1 mM) like putrescine, spermidine, spermine along with plant growth regulators auxin (IAA — 1.5 mg/L, IBA —1 mg/L) and cytokinin (KIN — 2 mg/L, BAP — 1.5 mg/L) to induce calli, direct shoot organogenesis and multiplied axillary shoot formation. Their HPLC study of the metabolite from in vitro regenerated plant extracts as well as mother plant illuminated the fact that aristolochic acid was higher in in vitro roots than plants grown under natural field conditions thus corroborating the pivotal role of polyamines in augmenting the concentration of aristolochic acid in combination with phytohormones.

Conclusions and future prospective

To combat the increasing demands of *Aristolochia* phytochemicals, sustainable conservational strategies are needed to be implemented. Several biotechnological interventions are already being performed to preserve the germplasms and produce economically important phytochemicals of

these genera. Studies elucidating the use of different markers (morphological, biochemical, molecular) to identify and assess variability among several species of *Aristolochia* are of prime use. This review is a small effort to gather the existing information and state the knowledge gaps for future works.

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Declarations

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare no competing interests.

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Authors and Affiliations

Shreya Nath[1] · Nilanjana Ghosh[1] · Tauhid Alam Ansari[1] · Avinash Mundhra[2] · Manoj Tukaram Patil[3] · Abhijit Mane[4] · Abilash Valsala Gopalakrishnan[5] · Md. Habibur Rahman[6] · Manoj Kumar[7] · Radha[8] · Mimosa Ghorai[9] · Subhabrata Paul[1] · Abhijit Dey[9]

1 Institute of Health Sciences, Presidency University (Newtown Campus), Kolkata 700156, India

2 Department of Botany, Naihati, North 24 Parganas, West Bengal- 743165, Kolkata, India

3 Post Graduate Department of Botany, SNJB's KKHA Arts SMGL Commerce, and SPHJ, Science College Chandwad (Nashik) Maharashtra, Affiliated to Savitribai Phule Pune University, Pune, Maharashtra, India

4 Department of Zoology, Dr. Patangrao Kadam Mahavidhyalaya, Ramanandnagar (Burli) Affiliated to Shivaji University, Tal: Palus, Dist: Sangli, Maharashtra 416308 Kolhapur, India

5 Department of Biomedical Sciences, School of Bio Sciences and Technology, Vellore Institute of Technology (VIT), Vellore, Tamil Nadu 632014, India

6 Department of Global Medical Science, Wonju College of Medicine, Yonsei University, Gangwon-do, Wonju 26426, Korea

7 Chemical and Biochemical Processing Division, ICAR - Central Institute for Research On Cotton Technology, Mumbai 400019, India

8 School of Biological and Environmental Sciences, Shoolini University of Biotechnology and Management Sciences, Solan 173229, Himachal Pradesh, India

9 Department of Life Sciences, Presidency University, 86/1 College Street, Kolkata 700073, West Bengal, India