International Journal of Advanced Research in Engineering Technology & Science
ISSN: 2349-2819
Email: editor@ijarets.org Volume-5, Issue-4 April- 2018 www.ijarets.org

Genetic Improvement in *Boerhaavia diffusa* L. for Economic Traits: Strategies and Scopes

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Abstract

Boerhavia diffusa L. an herbaceous member of family *Nyctaginaceae*, is also known as Punarnava. *B. diffusa* is indigenous to India and found throughout the tropical and subtropical parts of the country. Due to its high medicinal properties and overexploitation this species rapidly diminishing from the nature and natural resources that why urgent need of new genotype/ variety for the fulfillment of the supply of raw material to the pharmaceutical company. In the absence of genetic variability for economic traits in the overexploited accessions, variability should be induced (mutagenized) in the identified promising accession which high economic yielding potential. TILLING (Targeted Induced Local Lesions In Genomes) is a reverse genetic strategy that utilizes chemical mutagenesis for inducing variability and sensitive molecular screenings to identify point mutations responsible for phenotype alteration. This review suggested that due to creation of genetic variability through mutation breeding quantitative characters will be developed which are controlled by multiple genes or polygenes, (QTL genes) are influenced by environmental factors.

Key words: *Boerhavia diffusa*, *H*epatoprotective, Antidiabetic, Punarnavine

Introduction

Boerhavia diffusa L. an herbaceous member of family *Nyctaginaceae*, is also known as Punarnava, Raktapunarnava, Shothaghni, Kathillaka, Kshudra, Varshabhu, Raktapushpa, Varshaketu amd Shilatika in India (Yelne *et al*., 2000). *B. diffusa* is indigenous to India and found throughout the tropical and subtropical parts of the country up to an altitude of 2000 m in the Himalayan region (Dhar *et al*., 2011). Out of the 40 species of this genus, six are found in India e.g., *B. diffusa, B. chinensis, B. erecta, B. repens, B. rependa*, and *B. rubicunda*. The preliminary screening of the *B. diffusa* plant revealed the presence of sugars, sterols (Singh and Udupa, 1972), β-sitosterol (Srivastava *et al*., 1972), alkaloids (Garg *et al*., 1980; Shukla, 1982) and alkaloids (0.04%) known as punarnavine and punarnavoside (Surange and Pendse, 1972). Hentriacontane, β-

sitosterol and ursolic acid along with glucose, fructose and sucrose isolated from the roots (Misra and Tiwari, 1971), new dihydro-isofuranoxanthone (methyl 3, 10-dihydro-11-hydroxy-1-methoxy-4,6-dimethyl-10-oxo-1*H*-furo [3,4-*b*]xanthene-3-carboxylate) isolated from the benzene extract of the roots and designated as borhavine (Ahmed and Yu, 1992), and C-methyl flavones(5,7-dihydroxy- 6-8-dimethoxy flavones) from root (Gupta and Ahmad, 1984), rotenoids also known as boeravinones (boeravinone A-F) isolated from the roots (Misra and Tewari, 1971; Jain and Khanna, 1989; Kadota et al., 1989; Lami *et al*., 1992) and boerhavisterol (9,10-seco-stigmast-5,8 (9)- dien- 3β-ol), boerhadiffusene (1-(2',6',6'-trimethylcyclohex-1'-enyl) -11-(3"-3"-dimethyl cyclohexyl) -4,8-dimethy l–undiec-1-ene), diffusarotenoid (4,9- dihydroxy- 10-methyl- 6a-dehydrorotenoid-6-pentanoate), boerhavilanastenyl benzoate (27-*O*- (4'-benzoyl-β-Dglucopyranosyl) 9β-lanost-5-en-3-one) and a rotenoid, boerhavinone A (6-methoxy-9,11- dihydroxy- 10-methyl-6-a, 12a-dihydrorotenoid) isolated from root (Gupta and Ali, 1998). Two lignans, liriodendrin and syringaresinol mono-beta-D-glucoside, have been isolated from the methanol extract of the roots and the former compound was found to exhibit a significant calcium (Ca2+) channel antagonistic effect in frog heart single cells (Lami *et al*.,1991).

According to literature survey only some biochemical compounds such as alkaloid and flavonoid have been characterized in *B. diffusa* but no information available in the area of genetic improvement for development of high yielding genotype/chemotype which is a gap present in this crop. Keeping these gaps in mind, a systematic R & D activity is urgently required on its genetic improvement for the development of high yielding genotype/chemotype so that continuous supply of quality due to daily use as ethano-botanical by villagers, tribal peoples, pharmaceutical drug formulations by pharmaceutical industry, the plant *B. diffusa* gradually depleting from the nature and natural resources due to overexploitation, even not little bit work is going on its genetic improvement for superior chemo-morpho-genotype so this reviews is promising to improving better genotype/ variety for future fulfillment of raw materials for pharmaceutical company.

Global scenario of raw material of medicinal plants

Presently, 95% raw materials required by pharmaceuticals and drug manufactures are collected from the wild and remote areas (Kehimker, 2000). Raw material may be the any parts of plants viz leaves, roots, fruits, bark, stems, rhizomes, seeds, flowers, plant juices, extract or whole plant. Throughout the world demand for the plant-based medicines is increasing due to their safety, quality and effectiveness. WHO estimated that 50% population of developing countries rely on traditional medicine mostly plant drug for their primary health care and present demands is approximately US$ 14 billion per year (Kala, 2006, Sharma 2010)? India is also

a major exporter of medicinal plant raw materials and their extracts. The country exported a total of 42000 tones of medicinal plant raw materials to other countries during the year 2000-2001 (Sarin, 2003). Medicinal plants occupied an important position in the socio-cultural, spiritual and medicinal arena of rural people of India. World Health Organization **(**WHO) estimated that 80% of the population of developing countries rely on traditional medicine mostly plant drugs, for their primary health care needs (Anonymous, 2012). The domestic trade of Ayush industry is the order of Rs. 80-90 billion (IUS$= Rs 50). The Indian medicinal plants and their products accounts for exports in the range of Rs.10 billion dollar per annum. There is global resurgence in TSM resulting in increased world herbal trade which stands at US$ 120 billion and is expected to reach US$ 7 trillion by 2050. In case of Western medication is mainly associated with the different severe side effects and high costs of drugs. The most common and effective antidiabetic medicinal plants of Indian origin are Giloe (*Tinospora cordifolia*), gurmar (*Gymnema sylvestre*), garlic (*Allium sativum*), Beetroot (*Beta vulgaris*), methi (*Trigonella foenum-graecum*), ghrita kumara (*Aloe vera*), neem (*Azadirachta indica*), ash gourd *(Benincasa hispida*), tulsi (*Ocimum sanctum),* anar (*Punica granatum*), purging Nut (*Jatropha curcas*), fever nut (*Caesalpinia bonducella*), bisasar (*Pterocarpus marsupium*), jamun (*Syzygium cumini*), karela (*Momordica charantia*), bael (*Aegle marmelose*), church steeples (*Agrimonia eupatoria*), mango (*Mangifera indica*), mulberry *(Morus alba*), Babul (*Acacia arabica*), onion (*Allium cepa*), bitter apple (*Citrullus colocynthis*), eucalyptus (*Eucalyptus globules*), banyan tree (*Ficus benghalenesis*), potato *(Ipomoea batatas*), kewach (*Mucuna pruriens*), gurhal (*Hibiscus rosasinesis*), ivy gourd (*Coccinia indica*).In India the medicinal plant related trade is estimated to be approximately US $ 1billion per year (Joshi *et al*., 2009). According to an estimate, the quantity of export of ayurvedic products produced in India has tripled between last two financial years. In 2008, India exported medicinal plants worth eight billion dollars, 60% was in crude form, while 30% was in the form of finished products. Rest of them was partially prepared products (Malik *et al.*, 2011). The requirement of higher productivity of raw materials, secondary metabolite content, bioactive compounds, herbal tea, drugs and medicines, high yielding variety for biotic and abiotic stress conditions, herbage product not enough as in Indian population ratio. The medicinal properties of plant species have made an outstanding contribution in the origin and evolution of many traditional herbal therapies and B. *diffusa* is an herb and herbal demand is increasing day by day Many medicinal plants in India are restricted to the traditional and ethno botanical uses among tribal's and villagers. Due to population demand, it is necessary to know the scientific basis of the chemical constituents present in ignored medicinal plant species which suggesting to the pharmaceutical company for provide drugs and medicines for their use and exploitation. There are many other potential causes of rarity in medicinal plant species, such as habitat specificity, narrow

range of distribution, land use disturbances, introduction of non-natives, habitat alteration, climatic changes, heavy livestock grazing, explosion of human population, fragmentation and degradation of population, population bottleneck, and genetic drift (Kala 2000,2005, Weekley and Race 2001). More recently, the publication of red data book by IUCN as well as proceedings of a few regional meetings on this topic have helped to understand the relative abundance or scarcity of various medicinal plant species including the rare, threatened, endangered, or species about to become extinct (Salleh *et al*. 1997; Anon 1998; Gautam *et al*. 1998; Tandon *et al*. 2001). Recently, some rapid assessment of the threat status of medicinal plants using the IUCN-designed Conservation Assessment and Management Plan (CAMP) methodology revealed that about 112 species in Southern India, 74 species in Northern and Central India. It is therefore necessary to collect, conserve and evaluate germplasm and to develop agro technologies for medicinal and aromatic plants with potential for farming. Owing to the need and global resurge of herbal medicine creates a huge pressure on the plant population which is naturally grown in the forest used for the pharmaceutical industries, *B. diffusa* plant becomes endangered. Keeping its medicinal importance and threatened status of the plant, need to start genetic improvement work on it and develop superior and suitable genotype/mutant/chemovar/varieties for commercial cultivation so that its regular supply becomes ensured to the pharmaceutical industries. *Boerhavia diffusa* Linn., is an important medicinal plant having alkanoids, flavonoids used in various human diseases. There are few scarcely reports are available on its chemical characterization and cytological studies but there is no reports available on its genetic improvements for economic traits.

Consequences and future prospectus of creating genetic variability.

To speed up genetic improvement for the release of high economic yielding genotypes/chemovars/varieties are the main output of this review. These high yielding genotype/chemovar/varieties solve the problems of the global demand to producing herbal products for drugs and pharmaceutical industries such as Lee 52 by Himalya Company, etc. In absence of desirable variants among available genetic stock *B. diffusa* of phenological changes which are useful have been induced in identified accessions/ genotypes by mutagen treatment including, disease resistance, early maturing, changes in leaf shape and size, increased number of flowers and flower colours. Due to changes in petal color, the flavonoid content as well as new flavonoids and alkanoid automatically increases in the roots OF *B. diffusa* Male sterility is also develop which help in cross pollination and increasing heterozygosity. The structural and functional genomics research on cereal genomes, which during the past two decades has covered both basic and applied aspects, deepens our understanding about gene networks for chemovar development and economic triais through the available

molecular maps, genomic and expressed sequence tag (EST) sequences and the interaction of gene products, and information about QTLs (quantitative trait loci or genomic regions that are associated with a phenotypic trait exhibiting continuous variation)TILLING consists of mutagenesis, DNA isolation and pooling, and high-throughput mutation discovery in targeted genes. First described in Arabidopsis (McCallum et al., 2000) and Drosophila spp. (Bentley et al., 2000), it has been successfully extended to multiple model and economic species, thus becoming an important tool for functional genomics. Originally, TILLING discovered mutations through the detection of mismatched sites in PCR products (Oleykowski et al., 1998; Till et al., 2004a; Dong et al., 2009). The advent of low-cost high-throughput sequencing has added another powerful method (Rigola et al., 2009; Tsai et al., 2011)

Work carried out in *Boerhaavia diffusa*

The active principle contained in the herb is an alkaloid, known as punarnavine, an alkaloid from *B. diffusa* could enhance the immune response against metastatic progression of B16F-10 melanoma cells in mice (Chopra 1969) Eupalitin-3-O–*β*-d-galactopyranoside (Bd-1) isolated and purified from the ethanolic leaf extract of *B. diffusa* shows selective immunosuppressive activity (Agarwal et al 1936) The roots and leaves with flowers have been found to be highly potent (CSIR, 1988). In Ayurvedic medicine, different parts of this plant were reported to have various medicinal properties. It was used in renal ailments as diuretic (Anand, 1995); and to treat seminal weakness and blood pressure (Gaitonde *et al.*, 1974). It is also used in the treatment of stomach ache, anemia, cough, and cold, and as a diaphoretic, laxative, expectorant, and a potent antidote for snake and rat bites (Chopra *et al.*, 1956), in the treatment of nephrotic syndrome (Singh and Udupa, 1972), hepatitis, gall bladder abnormalities, and urinary disorders (Mudgal, 1975; Cruz, 1995). The flowers and seeds are used as contraceptive (Chopra *et al.*, 1956). Pharmacological studies have demonstrated that *punarnava* possesses punarnavoside, which exhibits a wide range of properties – diuretic (Gaitonde *et al*., 1974); anti-inflammatory (Bhalla *et al*., 1968); antifibrinolytic (Jain and Khanna, 1989); anticonvulsant (Adesina, 1979); antibacterial (Olukoya *et al*., 1993); antistress agent; antihepatotoxic (Mishra, 1980; Chandan *et al.*, 1991; Rawat *et al.*, 1997); anthelmintic febrifuge, antileprosy, anti-asthmatic, antiscabies, and anti-urethritis (Nadkarni, 1976); and antinematodal activity (Vijayalakshmi *et al*., 1979). An aqueous extract of thinner roots of *B. diffusa* at a dose of 2 ml kg-1 exhibited marked protection of various enzymes such as serum glutanic oxaloacetic transaminase, serum glutanic-pyruvic transaminase, and bilirubin in serum against hepatic injury in rats (Rawat *et al.*, 1997). *Punarnava* possesses diuretic and anti-inflammatory activities and the maximum activity was observed in samples collected in the rainy season. Due

to the combination of these two activities, *punarnava* is regarded therapeutically as highly efficacious for the treatment of inflammatory renal diseases and common clinical problems such as nephrotic syndrome, oedema, and ascites resulting from early cirrhosis of the liver and chronic peritonitis. The plant is reported to be efficacious in abdominal tumors and cancers. The drug proved useful as a hematinic and as a growth promoter in children fed with milk fortified with the drug. In the form of a powder or an aqueous decoction, the drug was found to be useful in the treatment of nephritic syndrome and compared well with corticosteroids. The drug decreased the albumin urea; the serum protein was increased and serum cholesterol level was lowered. Singh and Udupa (1972) reported that dried root powder showed curative efficiency when administered orally for one month to children or adults suffering from helminth infection. The subjects became worm-free within five days of treatment. The drug, singly or in combination with other drugs, was found to be effective in liver disorders, heart diseases (hypertension, angina, cardiac failure, etc.), respiratory tract infections, leukorrhea, spermatorrhea, etc. The purified glycoprotein from *B. diffusa* exhibited strong antimicrobial activity against RNA (ribonucleic acid) bacteriophages (Awasthi and Menzel, 1986). With much of the clinical research validating its long history of different uses in natural medicine, the commercial bulk of *punarnava* in India represents heterogeneous medicinal uses. The entire plant including the roots is eaten as vegetable, in curries and soups. The roots and seeds are added to cereals, pancakes, and other foodstuffs. They are also served as bird feed or poultry feed. The plants are grazed by sheep, goats, and cows, and in West Bengal, it is believed that the plant enhances lactation period and also the amount of milk in cattle (CSIR, 1988). In view of the pharmacological, clinical, and medicinal potential of this plant, the authors screened the root, leaf, stem, flower, and seed samples (collected at different stages of plant growth and from different locations, both fresh and dried) for their antiviral activity against a number of isometric as well as anisometric phytopathogenic viruses, in various host/virus combinations both in vitro and in vivo (Verma and Awasthi, 1979). Maximum antiviral activity, in each case, was recorded with the aqueous extract of dried root powder applied before virus inoculation. The active principle was purified and isolated (Verma *et al*., 1979). The roots of *B. diffusa* are a rich source of a basic protein, which is used for inducing systemic resistance in many susceptible crops against commonly occurring viruses (Verma and Awasthi, 1979; 1980; Verma *et al*., 1979; Awasthi *et al.* 1984; 1985; 1989). This protein or antiviral agent was active against tobacco mosaic virus in *Nicotiana glutinosa*, *Datura metel*, *Chenopodium amaranticolor*, and *Nicotiana tabacum* (Ky58 White Burley and NP31); sunnhemp rosette virus in *Cyamopsis tetragonoloba*, *Vigna unguiculata*, and *Crotalaria juncea*; and gomphrena mosaic virus in *Chenopodium amaranticolor*, *Vigna unguiculata*, and *Gomphrena globosa* when applied a few hours (2–24 h) before inoculation by the respective inocula of viruses (Verma and Awasthi,

1979; Awasthi *et al.*, 1984). The antiviral agent was a basic glycoprotein (70–80% protein and 8–13% carbohydrates) with a molecular weight of 16–20 kDa as determined by gel filtration chromatography (Verma et al., 1979). The resistance-inducing protein was found to be extremely thermostable (Verma and Awasthi, 1979). Following treatment with the systemic resistance inducing protein, the susceptible healthy host produced a virus inhibitory agent (VIA). The VIA showed the characteristics of the protein, and upon incubation with the virus, reduced infectivity of the viruses both in vitro and in vivo (Verma and Awasthi, 1980). Upon gel filtration on Sephadex G- 75®, two active fractions, exhibiting protein characteristics, were recovered (Verma and Awasthi, 1980, Awasthi *et al*., 1987). The VIA was present both in treated as well as untreated leaves. The biophysical characteristics of induced VIA were also studied and it was found to be a basic protein (Awasthi *et al*., 1987). The glycoprotein occurring in *B. diffusa* roots functions as a signal molecule, and is of great interest as it has a role in stimulating the defence systems of plants against viruses. Owing to the high antiviral efficacy of *B. diffusa* under laboratory conditions, it was tested under field conditions as well against a few viral diseases of economically important crops. The purified glycoprotein from *B. diffusa* reduced infection and multiplication of tomato yellow leaf curl virus (Awasthi and Rizvi, 1999), papaya ring spot virus (Awasthi, 2000), and cucumber green mottle mosaic virus (Awasthi *et al.*, 2003). The aqueous crude extract from the dried roots was also found significantly active against a number of viruses – mung bean yellow mosaic virus (Awasthi, 2000); bean common mosaic virus (Singh and Awasthi, 2002); water melon mosaic virus (Awasthi, 2002); bottle gourd mosaic virus in muskmelon (*Cucumis melo*), ridged gourd (*Luffa acutangula*), and bottle gourd (*Lagenaria siceraria*) (Awasthi and Kumar, 2003); cucumber mosaic virus in cucumber (*Cucumis sativus*) and muskmelon and water melon mosaic virus in watermelon (*Citrullus lanatus*) (P Kumar, personal communication); and mung bean yellow mosaic virus in mung bean (*Vigna radiata*) (S Singh, personal communication). In Purulia (West Bengal), tribals eat this plant as vegetable. *Boerhaavia* leaves are cooked and eaten in Assam, where it is commonly found in the markets. Its roots are used in the treatment of piles by the inhabitants of the Garhwal Himalaya (Uttaranchal). The root paste is used to cure bloody dysentery by the Bhils of the Jhabua district in Madhya Pradesh. The decoction of the plant is given in the treatment of nodules in the body. The root juice is used in treating asthma, scanty urine, and internal inflammation disorders. *B. diffusa* is used for curing ailments such as leukorrhea, rheumatism, and stomach ache by the Sahariya tribe in the Lalitpur district of Uttar Pradesh. This plant is also used by the tribes of Ambikapur district (Madhya Pradesh) for the treatment of elephantiasis. In the Indo-Nepal Himalayan terai region, the tribals harvest this plant for medicinal purposes, mainly for flushing out the renal system, and to

treat seminal weakness and blood pressure (Mitra and Gupta, 1997). The *B. diffusa* plant contains a large number of such compounds as flavonoids,alkaloids, steroids, triterpenoids, lipids, lignins, carbohydrates, proteins, and glycoproteins. Punarnavine C17H22N2O mp 236–237°C (Agarwal and Dutt, 1936; Basu *et al.*, 1947; Surange and Pendse, 1972), boeravinone A.F (Kadota *et al.*, 1989; Lami *et al.*, 1990; 1992), hypoxanthine 9-L-arabinofuranoside (Ahmad and Hossain, 1968), ursolic acid (Mishra and Tiwari, 1971), punarnavoside (Jain and Khanna, 1989), liirodendrin (Aftab *et al.*, 1996), and a glycoprotein having a molecular weight of 16–20 kDa (Verma *et al.*, 1979) have been isolated and studied in detail for their biological activity.

Chopra *et al.* (1923) reported that the plant contained large quantities of potassium nitrate, besides punarnavine. The herb and roots are rich in proteins and fats. The herb contains 15 amino acids, including 6 essential amino acids, while the root contains 14 amino acids, including 7 essential amino acids. Seth *et al.* (1986) isolated a new anti-fibrinolytic compound 'punarnavoside' from the roots of *B. diffusa*. Phytochemical screening of the roots from garden-grown in vivo plants of *B. diffusa* of different ages revealed that the maximum alkaloid content (2%) accumulated in the roots of 3-yearold mature plants. Different parts of the *B. diffusa* plant have been widely used by indigenous tribes in the traditional system of medicine. The roots have been widely used for the treatment of dyspepsia, jaundice, enlargement of spleen, abdominal pain, abdominal tumors, and cancers (Kirtikar and Basu, 1956).

The root powder, when mixed with *mamira* (*Thalictrum foliolosum*), is used to treat eye diseases. It cures corneal ulcers and night blindness (Gupta *et al.*, 1962), and helps restore virility in men. People in tribal areas use it to hasten childbirth (Shah *et al.*, 1983). The juice of *Boerhavia* leaves serves as a lotion in ophthalmia. It is also administered orally as a blood purifier and to relieve muscular pain. In old Indian books of medicine such as the Charaka Samhita and Sushrita Samhita, it is mentioned that the Ayurvedic preparations made from *punarnava* – namely, *punarnavastaka kvath*, *punarnavakshar*, and *punarnava taila* – were used for the treatment of various ailments. The whole plant of *B. diffusa* is a very useful source of the drug *punarnava*, which is documented in Indian Pharmacopoeia as a diuretic (Chopra, 1969). Two rotenoids isolated from *B. diffusa*, boeravinones G and H, have been found to potently inhibit the drug efflux activity of breast cancer resistance protein (BCRP/ABCG2), a multidrug transporter responsible for cancer cell resistance to chemotherapy (Ahmed et al., 2007).

Approaches and functional genomics of studies in mutation breeding

Molecular markers: A molecular marker (or genetic marker) is a specific fragment of DNA sequence that is associated to a part of the genome and can be identified within the whole genome. Molecular markers are used to 'flag' the position of a particular gene or the inheritance of a particular characteristic.

RFLP: Restricted fragment length polymorphisms. This is one of the earliest type of DNA marker. DNA is cleaved with restriction enzymes that recognize specific sequences in the DNA, the resulting fragments are separated by size using electrophoresis. Probes that are generated from specific regions of the genome are used to identify fragments corresponding to the target sequence. PCR-based markers

RAPD: Randomly amplified polymorphic DNAs. Sections of DNA are randomly amplified from different regions of the genome using PCR primers of approximately 10 bp (base pairs) of arbitrary sequence to generate a range of markers, normally visualized as banding profiles in gels after electrophoresis.

SSR: Simple sequence repeats, also known as microsatellites. SSRs are tandemly repeated nucleotides of 1-4 bp in size, e.g., mononucleotide repeats, (A)n, (T)n, (G)n and (C)n; bi-nucleotide repeats such as (CT)n, (AG)n, tri-nucleotide repeats such as (TCT)n, (AGA)n, etc. Polymorphism is based on differences in the number of repeats (length of repeated sequence).

EST-SSR: SSR located in expressed sequence tags (ESTs). ESTs are directly related to gene expression and EST libraries can be developed for specific plant tissues (e.g. root ESTs) and responses to specific environments (e.g. heat stress). EST-SSRs are especially useful for comparative mapping and evolutionary studies because they have a high likelihood of being syntenic between species.

Indel: DNA insertion/deletion. Indels are polymorphisms of short insertions and deletions, which can be spread across the genome, and therefore recognised as an abundant source of genetic markers, though not as common as SNPs. In most of cases, Indels are referred to as non-repetitive sequences.

ISSR: Inter simple sequence repeat. As the name implies ISSRs are genomic regions located between SSR sequences. These regions can be amplified by PCR using simple repeat sequences as primers. Various SSR sequences can be anchored at the 3'-end of the primer to increase their specificity (usually to reduce the number of bands on a gel to a manageable number). ISSRs are mostly dominant markers, though occasionally a few may exhibit co-dominance. An unlimited number of primers can be synthesized for various combinations of di-, tri-, tetra- and penta-nucleotides.

SNP: Single nucleotide polymorphisms. SNPs are single base changes in the DNA sequence that are generally bi-allelic at any particular site. SNPs can be revealed through various approaches, but sequencing methods are rapidly being developed that aid SNP detection.

SRAP: Sequence-related amplified polymorphism. SRAP is based on two-primer amplification. The primers are 17 or 18 nucleotides long and consist of the core sequences and selective sequences. The core sequences are 13 to 14 bases long, of which the first 10 or 11 bases at the 5 end are sequences of no specific constitution (known as "filler" sequences), followed by the sequence CCGG in the forward primer and AATT in the reverse primer. The core is followed by three selective nucleotides at the 3 ends. The filler sequences of the forward and reverse primers must be different from each other and can be 10 or 11 bases long. In the PCR, the annealing temperature for the first five cycles is set at 35°C. The following 35 cycles are run at 50°C. The amplified DNA fragments are separated and detected by denaturing acrylamide gels.

ASAP: Allele-specific associated primers. Specific primers are designed according to sequence information of specific alleles and used for amplification of DNA to generate a single fragment at stringent annealing temperatures. They also known as allele-specific or gene specific markers.

VNTR: Variable number of tandem repeats. VNTRs are also known as minisatellites that are tandem repeats with a monomer repeat length of about 11–60 bp. The minisatellite loci contain tandem repeats that vary in the number of repeat units between genotypes and are referred to as variable number of tandem repeats or hypervariable regions (HVRs). Combination of restriction and PCR

AFLP: Amplified fragment length polymorphism. This technique combines aspects of RFLP and PCR amplification. The technique is based on the detection of genomic restriction fragments by PCR amplification and can be used for DNAs of any origin or complexity without any prior knowledge of sequence, using a limited set of generic primers. The number of fragments detected in a single reaction can be 'tuned' by selection of specific primer sets.

CAPS: Cleaved amplified polymorphic sequence. This technique combines PCR amplification and restriction enzyme digestion to generate DNA polymorphisms. Polymorphic patterns are generated by restriction enzyme digestion of PCR products. PCR primers for this process can be synthesized based on the sequence information available in data bases of genomic or cDNA sequences or cloned RAPD bands. These markers are co-dominant in nature.

STS: Sequence-tagged sites. RFLP probes specifically linked to a desired trait are sequenced and converted into PCR-based STS markers based on nucleotide sequence of the probe resulting in a polymorphic band pattern for the specific amplicon. In this technique the tedious hybridization procedures involved in RFLP analysis can be overcome.

Serial analysis of gene expression (SAGE): is a technique designed to take advantage of high-throughput sequencing technology to obtain a quantitative profile of gene expression that measures not the expression level of a gene, but quantifies a 'tag' that represents the transcriptome product of a gene. A tag, for the purpose of SAGE, is a nucleotide sequence of a defined length directly adjacent to the restriction site for a particular restriction enzyme closest to the 3' end. The data product of the SAGE technique is a list of tags with their corresponding count values – a digital representation of cellular gene expression. Based on the length of tags, several modified forms of SAGE, such as, MicroSAGE, MiniSAGE, LongSAGE and SuperSAGE, have been developed.

Quantitative trait locus (QTL): QTL analysis is a statistical method that links two types of information phenotypic data (trait measurements) and genotypic data (usually molecular markers) in an attempt to explain the genetic basis of variation in complex traits. QTL analysis allows researchers in fields as diverse as agriculture, evolution, and medicine to link certain complex phenotypes to specific regions of chromosomes. The goal of this process is to identify the action, interaction, number, and precise location of these regions.

Tiling microarray: involves the generation and immobilization on a glass slide of nucleic acid probes that represent a target genomic region. These probes can either overlap, lay end-to-end or be spaced at a predefined average distance in genomic space. A sequence of probes spanning a genomic region is called a 'tile path', or a 'tiling', and the average distance, in nucleotides, between the centers of neighboring probes is termed the 'step' or 'resolution' of the tiling. Each probe on a tiling array interrogates the presence of a sequence in a nucleic acid population by hybridization. Tiling arrays can be developed for sequenced organisms only, and among plant species, Arabidopsis and rice enjoy the development and application of such arrays.

Tilling: Targeting-induced local lesions in genomes is a reverse genetic method that combines random chemical mutagenesis with PCR-based screening of genes of interest. This provides a range of allele types.

Knock-Out Mutations: By comparing the phenotypes of isogenic genotypes differing in single sequence motifs, TILLING provides direct proof-of-function of both induced and natural polymorphisms without the use of transgenic modifications.

Cytogenetic and molecular cytogenetic tools used to identify chromosome translocation

Chromosome C-banding: Method of defining chromosome structure by differential staining (banding) of constitutive (C) heterochromatin regions with Giemsa.

Genomic *in situ* **hybridization (GISH):** GISH is a genomic probing technique and a molecular cytogenetic technique, which uses genomic DNA from the alien species as a probe in combination with an excess of unlabelled wheat DNA in the hybridization solution to block cross hybridizations. GISH analysis allows physical determination of alien chromosome segment and the break points in these translocations and an estimation of the sizes of the transferred segments of the alien species.

Fluorescence in situ **hybridization (FISH):** A molecular cytogenetic technique in which a deoxyribonucleic acid (DNA) probe is labeled with a fluorescent dye conjugate (that can be visualized under a fluorescence microscope) and then hybridized onto target DNA, usually chromosome preparations on a microscopic slide. FISH allows direct mapping of DNA sequences to chromosome, and has become an important technique in plant molecular cytogenetics research. It is used to map genes physically and precisely to a specific region of a chromosome and can enumerate chromosomes, and/or detect chromosomal deletions, translocations, or gene amplifications in cells.

Conclusions:

There is no attempt has been made on genetic improvement in *B. diffusa* through mutagenesis. Thus, Germplasm will be tested and high yielding genotype/chemovar/variety for economic trait should be developed for the purpose of proper and appropriate supply of raw materials will be fulfilled the requirement for Pharmaceuticals Company. Collected landraces/ accessions of *B. diffusa* will be morpho-chemically characterized and potent high yielding alkaloids containing punarnavine, BOERAVINONE-B COCCINEONE–E EUPALITIN EUPALITIN-3-O GALACTOSIDE genotypes/variety will be identified through conducting yield assessment trails.

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