TOWARDS BIO-EFFICIENT AND NON-INVASIVE VETIVER : LESSONS FROM GENOMIC MANIPULATION AND CHROMOSOMAL CHARACTERIZATION

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Abstract: Vetiver is a plant of choice for soil-water conservation and amelioration, and is also an important source of essential oil. However, depending upon specific application of vetiver, specific plant or root ideotype need to be identified from a range of genetic diversity that may be prevalent around its primary centre of origin. This can be best scored from segregating seed progenies. But seed forming vetiver may not be environment friendly since there is an apparent threat of vetiver invasion to the nontarget areas through water or air borne seed dispersal. Therefore, if non-seediness could be achieved in the selected seed forming types then we could realise the twin advantage of having a desired genotype in non-seeding form suitable for controlled cultivation. Polyploidisation realised after genomic duplication could severely affect fertility leading to increased sterility on account of disturbances in meiotic behaviour and deficiency in sexual reproduction, and at the same time enhance the biological efficiency with respect to growth behaviour and metabolic efficiency. With this background, the present study was pursued to realise artificial polyploidy in a seed forming clone of vetiver. The polyploids thus developed evinced not only high gametic and seed sterilty but were also accompanied by enhanced biological potential for the characters associated with increased cell size and physico-physiological efficiency. The study has far reaching implications in utilizing a given ideotype for efficient application of vetiver ecotechnology for its multifarious environmental and industrial applications.

Keywords : Non-seeding vetiver, non-invasive vetiver, gametic sterility, polyploidy, genomic multiplication, meiotic behaviour , bioefficiency, essential oil secretary sites

1 INTRODUCTION

Vetiver traditionally used for extraction of essential oil from roots has lately been extensively utilized for its multifarious environmental applications (Lavania and Lavania 2000, Lavania *et al.* 2004). Whereas for the former application there is a need to identify genotypes that produce high concentration and superior quality of essential oil in their roots (Lavania 2003), but for the latter plant types supporting low oil / virtually no oil in their roots is desirable which works as a deterrant to the community of local root diggers (Lavania and Kumar 1998). However, in both the situations the natural diversity occurring in this species could serve as a valuable resource to isolate an ideal plant type with respect to root – ideotype (Lavania S 2003) suiting to specific needs for oil quantity and quality, or root physiography for environmental applications for soil and water conservation, pollution mitigation and soil - water detoxification.

India being the primary centre of origin and dispersion of *Vetiveria zizanioides* is rich for its genetic diversity (Lavania 2002). Some of the apparent indicators of genetic diversity attendant in vetiver in India are varition with respect to reproductive biology, flowering patterns, seed setting, and qualitative and quantitative differences in essential oil in the roots vis-à-vis chromosomal architecture in the various geographic zones within India (Lavania 1985). Whereas, exploration of core collections from different geographic zones could be used to isolate desired genotypes suiting to specific applications of vetiver, but seed forming vetiver prevelent in north Indian plains in wild state could be the suitable target to tap inherent natural diversity in segregating seed progenies for given applications. However, seed forming vetiver poses a potential threat of becoming weedy invading the nontarget areas on account of seed dispersal by air and / or water (Figure 1A). Therefore, if we have to realize the full potential of vetiver diversity, it is imperative that while identifying a desired genotype from amongst the natural diversity, we should also provide safeguards to mitigate indiscrimate spread of vetiver. Latter could be suitably pursued by minimising seed formation in the ideal genotypes isolated from seed forming wild types. The present investigation was therefore attempted to realise the following twin objectives:

(i) Realization of seed sterility in the seed forming genotypes to achieve non-invasiveness in vetiver
(ii) Realisation of enhanced biological potential in the given genotype.

2 MATERIAL AND METHODS

A seed-forming clone of vetiver "Kesari" (2n=20) isolated at the Central Institute of Medicinal and Aromatic Plant, Lucknow, India was considered for change into a seed-sterile type. "Kesari" is a profuse seed forming clone supporting very high pollen fertility (i.e >95 %) and seed germination of over 50%.

2.1 Genomic manipulation for induction of polyploidy

Seeds soaked in water overnight were sown in Petri-plates, and seedlings with about 2 cm long leaves were isolated for further experimental treatments. Outer layer of leaves in the seedlings was removed to expose the axillary buds, surface dried with soaking paper and then immersed in 0.1 % aqueous solution of colchicine in 2% DMSO for 7 to 9 hours in different batches at 25ºC, followed by thorough washing overnight in running water. The treated seedlings were subsequently sown in nursery beds, and further scored for stomatal guard cells after one month of growth. The seedlings that evinced enlarged stomatal guard cells were isolated and allowed to grow further, and subsequently examined after another one month for size of stomatal guard cells. Three different combinations of stomatal size from leaf peels were encountered i.e. stomatal size representing the parental stock, streaks of large and parental size of stomata i.e. mixoploid, and those showing enlarged stomatal guard cells throughout the entire leaf roughly double the size of parental stock. The latter were isolated, screened cytologically for doubled chromosome number i.e. genomic doubling (4x=40), and those showing consistency for duplicated chromosome number clonally multiplied to isolate stable polyploid forms.

2.2 Cytological analysis

Standard aceto-carmine squash technique (Sharma and Sharma 1980) was followed for both mitotic and meiotic chromosome analysis. However, since the roots are very hard in this species, therefore, the roots were first hydrolyzed in 1N. HCl at 60ºC for 10 minutes and then stained in 2% aceto-carmine for a week. For meiotic analysis, flower buds were fixed in Carnoy's fixative between

0800 to 0900 hrs to arrest various meiotic stages. At least five anthers representing different florets were used to perform meiotic analysis for meiotic configurations in both diploids and derived tetraploids. Pollen grains from anthers at pre-anthesis stage were excised and stained in aceto-carmine to ascertain pollen fertility. Those showing filled tendency and stained red are considered fertile and unstained and empty ones are taken as fertile.

2.3 Root anatomy, stomatal features and essential oil concentration

Hand-cut thin transverse sections of root double stained with fast green-safranine were studied under microscope for anatomical features. Attempts were made to identify essential oil storage cells by hydrolyzing the roots in 1N. HCL for 10 minutes followed by staining with Feulgen according to standard procedure that gave an indication of the localization of essential oil storage cells. The latter when studied under UV excitation evinced bright fluorescence for its cell wall. For leaf stomatal guard cells epidermal peels from the abaxial side were examined for the size of stomatal guard cells under light microscope as well as green light excitation that evinced red fluorescing chloroplasts in the stomatal guard cells. Observations were also recorded for the area covered by the essential oil secretary cells in the diploid *vs.* tetraploid, as well as on physical characteristics of roots in the diploid vs. tetraploid and the area covered by stomatal guard cells depicting photosynthetic efficiency.

3 RESULTS

3.1 Realization of stable polyploids

Although, it is quite difficult to realize artificial polyploidy in grasses on account of difficult to access merestematic region that lay deep seated beneath the leaf base, but concerted efforts exercised during the present experimentation, including use of career agents for targeted administration of colchicine have met with startling success in realization of polyploids in *Vetiveria zizanioides*. Further, in order to ascertain stabilization of polyploidy, lateral tillers were segregated, screened cytologically for polyploid status in the consecutive vegetative progenies. The isolated polyploids (4x=40) are cytologically stable for their somatic chromosome number. Experimental observations undertaken on diploid vs. tetraploid with respect to histo-morphological features, are given Table 1, and reproductive and meiotic behaviour are shown through Figure 1 and Figure 2, respectively. Root exomorphology and somatic chromosome number in diploid vs. tetraploid is depicted in Figure 3.

Table 1. Cell size associated changes in the diploid vs. tetraploid in vetiver with respect to bio-efficiency

Vetiver genomic status	Average root diameter (mm)	Average stellar diameter (mm)	Leaf epidermal features			Essential oil secretary cell physiography			% increase in secretary cell area in 4x
			No. of stomata / cm^2	Average size of single stomatal guard cell $(\mu m)^2$	% area covered under stomatal guard cells	No. of essential oil storage sites (phloem region) / root	Average cell size of essential oil storage group of phloem cells $(\mu m)^2$	% area under cover of essential oil storage cells*	
Diploid	1.6	1.05	14784	532	7.86	100	462	2.3	
Tetra-ploid	1.8	1.15	7524	1107	8.33	93	713	2.6	14.3

* does not include secretary cells of pith region

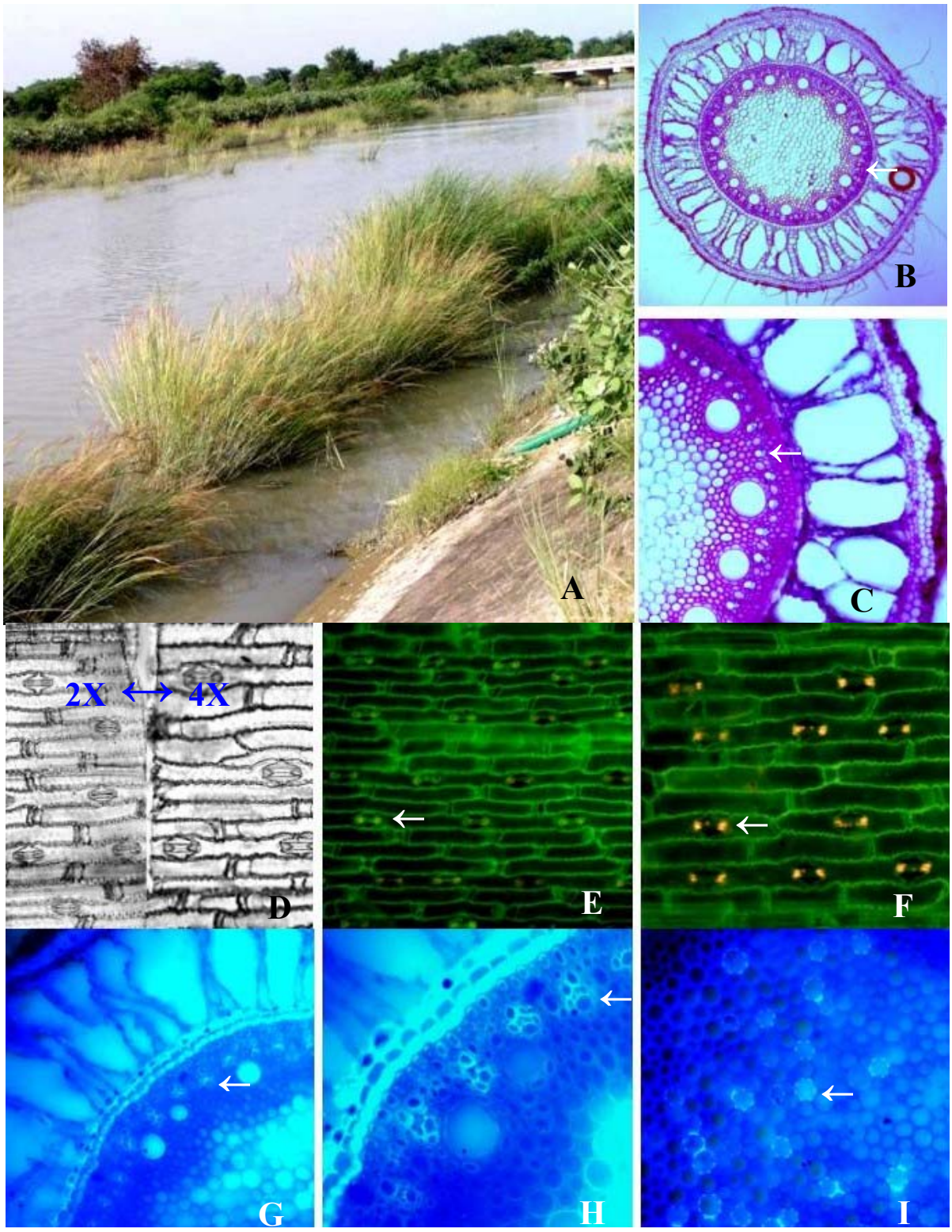

Figure 1. Vetiver habitat, and histo-morphological features in the diploid *vs*. autotetraploid. A. Natural dispersal of vetiver through irrigation canal depicting its weedy habit, B-C. Root cross section showing essential oil storage bast region, D. Side by side comparison of stomatal size in the diploid (left) and the tetraploid (right) – note enlarged stomata in the tetraploid, E-F. Leaf epidermal peel depicting red fluorescing chloroplasts in the diploid (E) and tetraploid (F), G-H. Root cross section showing blue fluorescing essential oil secretary bast region, I. Blue fluorescing secretary cells in the root pith region.

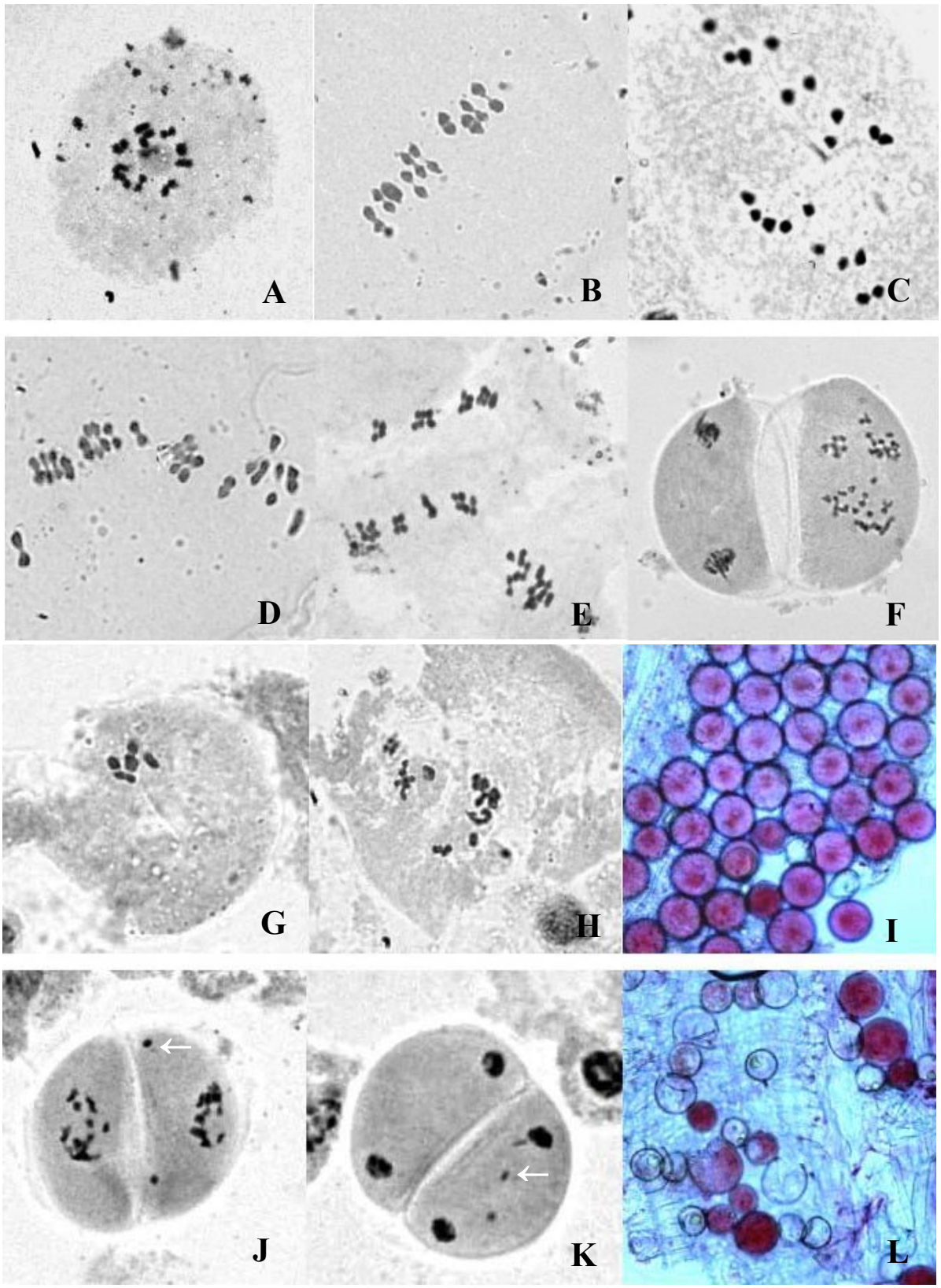

Figure 2. Meiotic behaviour and pollen fertility in the diploid (2n=2x=20) *vs.* autotetraploid (4x=40) in vetiver. A,B,C. Diplotene, metaphase and anaphase in the diploid showing balanced meiotic behaviour, D-F, and G,H, J,K. Meiosis in tetraploid : D. Occasional regular metaphase orientation in the tetraploid, E. Disturbed metaphase co-orientation, F. Imbalanced chromosome segregation during pollen formation G. Chromosome reduction, H. Disturbed polarity, J-K. Chromosome lagging during pollen formation. I-J. Pollen fertility in the diploid (I) and tetraploid (L) – note high pollen fertility in the diploid (I) in contrast to high pollen sterility in the tetraploid (L)

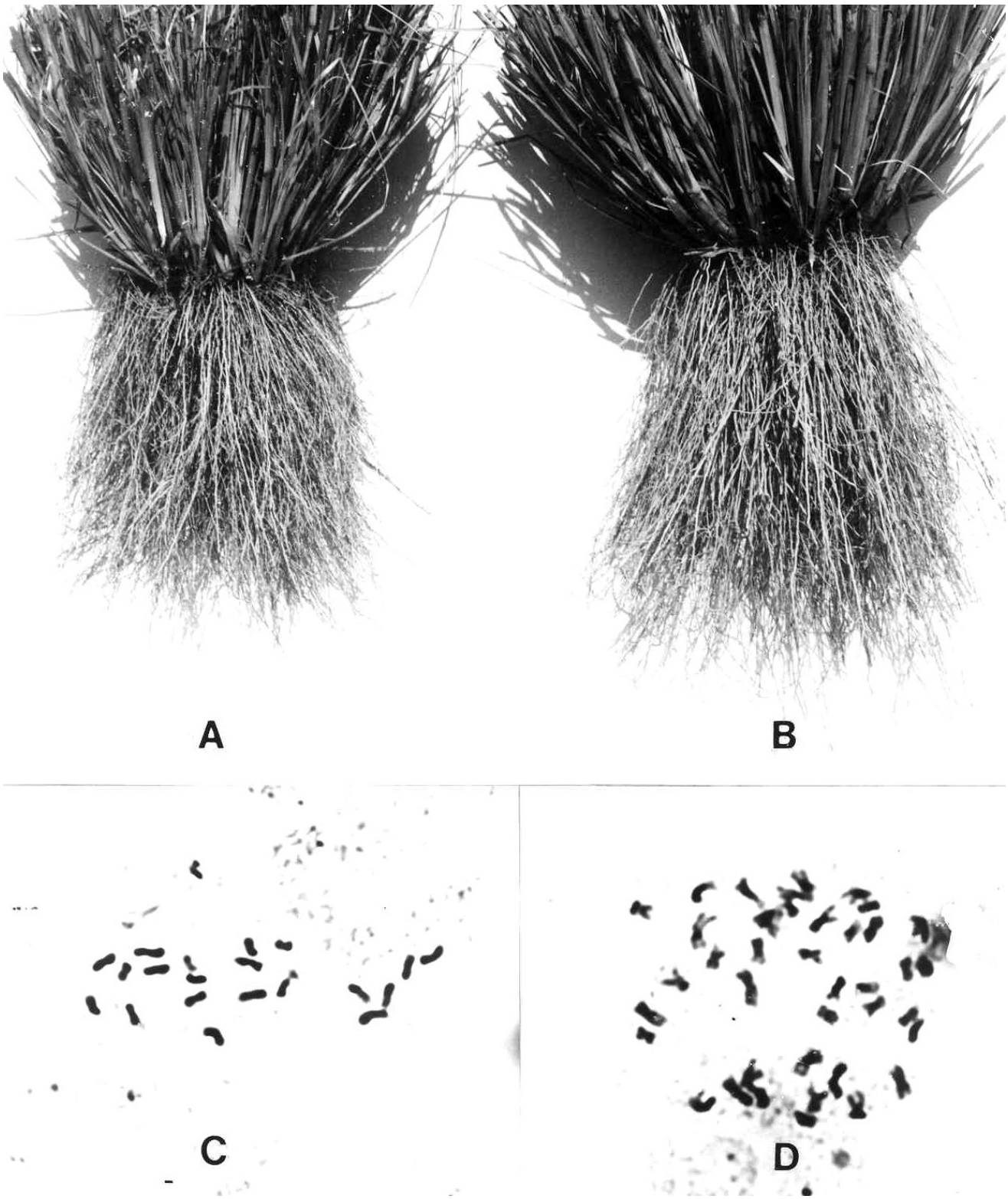

Figure 3. Comparison of root exomorphology in diploid (A) and tetraploid (B) and their respective somatic chromosome plates: C. Diploid (2n=2x=20) and D. Tetraploid (4x=40). Taken from an earlier publication of the senior author (Lavania 1988).

3.2 Exo-morphology and histo-morphological features in the source diploids vs. derived autotetraploids with respect to bio-efficiency

Vegetative tillers of equal size were grown under optimum cultural conditions to elucidate their relative growth behaviour and exomorphology. Data recorded on histo-morphological features is given in the Table 1. Essential oil secretary cells are localized in mature phloem region (Figure 1-G, H), and a few secretary cells were also present in pith region that evinced bright fluorescence under UV excitation (Figure 1- I).

3.2 Meiotic behaviour

Exhaustive meiotic analysis performed on the source diploid and the derived autotetraploid clearly reveal a balanced meiotic behaviour / anaphase segregation, and high pollen fertility in diploids (Figure 2- A,B,C and I) but highly disturbed meiotic behaviour and high pollen sterility in the tetraploids (Figure 2-D,E,F,G,H,J,K and L). Whereas normal meiotic features of organized metaphase orientation and balanced anaphase segregation capable of producing balanced gametes is observed in the diploids, but the same is totally lacking in the tetraploids. In the tetraploids metaphase orientation is highly disorganized leading to disturbed anaphase, lagging chromosomes and multi-polarity that lead to the formation of highly imbalanced gametes. This is clearly reflected in high pollen sterility i.e. > 90 % pollen sterility encountered in the tetraploids (Figure 2-L).

3.3 Reproductive behaviour and associated fertility

The vetiver clone undertaken in this study is high flowering and profuse seed forming clone. The pollen produced on the source diploid stock evince over >95 % pollen fertility followed by profuse seed formation. The seeds obtained from the diploid show over 50% seed germination under normal field grown conditions. On the other hand the tetraploids are late and low flowering, produce highly sterile pollen showing < 10 % filled pollen of variable size depicting high sterility (Figure 2-L). Less than 10% filled seeds are produced on the tetraploid, and they too are genetically imbalanced lacking germination potential and competitive growth.

4 DISCUSSION

4.1 Enhanced bio-efficiency of vetiver tetraploids

Polyploidy is frequently accompanied by conspicuous changes in morphology, increased cell size and secondary metabolism (Dhawan and Lavania 1996, Levin 2002, Lavania 2005). Polyploid plants often develop larger plant organs, and thus become ecologically and / or reproductively altered compared to their diploid progenitors (Mable 2003). The volume of teraploid cells is typically about double, and their surface area is about 1.5 times that of their diploid counterparts. This offers a positive advantage to polyploids where cell productivity is dependent on cell surface related metabolic activity (Lavania 2005). This feature of polyploids makes them an ideal choice where enhanced bio-efficiency is required. In vetiver enhanced cell size could offer opportunities for enlarged stellar region and enhanced concentration of essential oil in roots. Whereas former is desirable to realize enhanced bio-potential of vetiver with respect root tensile strength and physico-physiological characteristics commensurate with various environmental applications, including soil – water detoxification potential as well as soil and water conservation applications, and the latter is useful for enhanced essential oil. Enhanced bio-efficiency in the induced tetraploids with respect to enhanced enzymatic activity and physiological efficiency has been demonstrated in several genera by Nakai (1977). In accordance to the same, the present study is a pointer to that direction that polyploids in vetiver would evince enhanced bio-efficiency for various physico-physiological

characteristics, including root strength on account of thick stellar region / enhanced photosynthetic potential for increased chloroplast number in the stomatal guard cells / or enhanced essential oil productivity for increased cell area of secretary cells.

4.2 Realizing non-invasiveness in vetiver *via* genomic manipulation mediated seed sterility

Whereas a seed forming vetiver may serve as an ideal resource for isolating vetiver plant ideotype suiting to specific environmental / industrial needs, but the same seed forming property could pose a potential threat on account seed dispersal based invasiveness to non-target areas. As such, realization of non-invasiveness in seed forming vetiver is highly desirable for its eco-friendly environmental applications. Present study demonstrates that induced polyploidy besides enhancing the bio-potential of vetiver is also accompanied by high sterility. Latter adds value to realize non-invasiveness in vetiver since a desired seed forming genotype could be suitably converted into a non-seeding type. The non-invasive feature in the vetiver could be achieved on account of increased gametic / seed sterility encountered in the artificial polyploids on account of highly disturbed meiotic behaviour, imbalanced gamete formation leading to high gametic and seed fertility. Disturbed meiosis consummated through multivalent formation and resultant imbalanced anaphase segregation is quite common with artificial polyploids (Sybenga 1992). Although, tetraploids *per se* are found to be quite sterile in vetiver, but if we could achieve triploidy in vetiver by hybridizing tetraploid female with diploid male, it is likely that the triploids would offer not only triploid heterosis advantage with respect to plant bio-efficiency but also add on saving the plant resources from minimizing the energy wastage on flowering and other related activities. This opens newer opportunities on utilization of vetiver ideotypes.

5 CONCLUSIONS

The two most important objectives envisaged for the present study vis-à-vis eco-friendly utilization of vetiver were: (i) to realize non-invasiveness in vetiver, and (ii) to enhance the biological potential of vetiver. Both these objectives have been appropriately addressed in this communication. Although, natural genetic diversity could serve as a valuable resource for isolating desired genotypes, but latter could be quickly achieved if we tap the attendant variability from seed grown wild populations. However, in order to mitigate the weedy potential of such vetiver it is desirable to change the desired genotype into non-seeding one in order to utilize its full bio-potential in an eco-friendly sustainable manner. As such, in order to effectively implement the model objectives of this study, first we need to isolate a desired plant type from seed grown progenies from wild across the range of ecological diversity, and then experimentally change the same into a polyploid to make it non-invasive on account of attendant gametic and seed sterility. In the process the specific biological potential of the desired genotype would be commensurately enhanced on account of polyploidy, enhanced cell size and associated increased physico-physiological efficiency. This approach would have far reaching consequences to exhilarate isolation of ideal plant types suiting to specific environmental / industrial applications, and their effective implementation by growing in the target area without any fear of vetiver getting weedy or invasive to non-target natural habitats.

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Dr. UC Lavania, Dy. Director, associated with Central Institute of Medicinal and Aromatic Plants, Lucknow – a national laboratory of Council of Scientific and Industrial Research, Government of India, obtained his Ph.D. (1980) and D.Sc.(1998) in Botany from the University of Calcutta, Kolkata, India. He has been actively associated with vetiver research since 1985, and has worked extensively on cytogenetics and genetic improvement of aromatic grasses in general and vetiver in particular. Lately, in August 2005, he has guest edited Medicinal Plants Special issue of the journal *Plant Genetic Resources* published by CABI publishing, UK, and is the Managing Editor of the International journal *The Nucleus* . He has published over 80 research papers.