CYTOLOGICAL ANALYSIS OF ROOT CULTURES OF *Artemisia cina*

Tri Muji Ermayanti, Oktavia Yanti and Erwin Al Hafiizh

Research Centre for Biotechnology,
Indonesian Institute of Sciences (LIPI)
Jalan Raya Bogor Km. 46 Cibinong, Indonesia, 16911
Phone : 62-21-8754587; Fax. 62-21-8754588; E-mail : tmermayanti@hotmail.com

ABSTRACT

Artemisia cina is a medicinal plant species producing bioactive compounds which are potential as antitumor, antifungal and antibacterial. The aim of this study was to analyze the stability of chromosome numbers in root cultures of *A. cina.* Transformed root culture was established by infection of leaves of *A. cina* with *Agrobacterium rhizogenes* strains 07-20001, ATCC-15834, A4 and *A. tumefaciens* strain R1000. Roots isolated from glasshouse plants, plantlets grown in solid and liquid MS medium were utilized for investigation of chromosome examination of untransformed roots. Chromosome examination was conducted by squashing method and chromosome numbers were calculated under microscope. The results showed that both untransformed and transformed roots had instability in the chromosome number, but had the modal number of chromosome x=8 with the diploid number of 2n = 4x = 32. Roots isolated from glasshouse plants of *A. cina* had 53.7% of cells with the diploid numbers of 2n = 32, and 46.3% of cells had chromosome numbers ranged from 2n = 22 to 2n = 64. Untransformed roots isolated from plantlets cultured in solid media had only 36.1% of cells with chromosome number of 2n = 32, and untransformed roots grown in liquid medium had 49.4% of cells with 2n = 32. The chromosome numbers of *A. cina* transformed roots was affected by strains of *Agrobacterium.* Roots transformed with the bacterium strain 07-20001 showed the highest in normal chromosome numbers of 2n = 32 (62.4%) followed by roots transformed with strains ATCC-15834 (61.9%), R1000 (43.6%) and A4 (43.0%). The range of the chromosome number of untransformed roots was from 2n=17 to 2n=64, whilst that of transformed roots was from 2n=11 to 2n=66.

Keywords : untransformed roots, transformed roots, chromosome number, genetic stability, *Artemisia cina.*

ABSTRAK

Artemisia cina merupakan satu jenis tanaman obat yang memproduksi zat bioaktif yang potensial sebagai antitumor, anticendawan dan antibakteri. Tujuan dari penelitian ini adalah untuk menganalisis stabilitas jumlah kromosom pada kultur akar *Artemisia cina.* Kultur akar rambut diperoleh dengan menginfeksi daun tanaman dengan *Agrobacterium rhizogenes* galur 07-20001, ATCC-15834, A4 dan *Agrobacterium tumefaciens* galur R1000. Akar yang diisolasi dari tanaman yang ditumbuhkan di rumah kaca, planlet yang dikulturkan pada media MS padat dan kultur akar pada media cair digunakan untuk pengamatan kromosom untuk akar bukan hasil transformasi (akar normal). Pengamatan kromosom dilakukan dengan metode standar dengan pewarna asetoorsein. Setelah dihancurkan, akar diamati di bawah mikroskop. Hasil penelitian menunjukkan bahwa akar bukan hasil transformasi maupun akar rambut mempunyai ketidakstabilan dalam jumlah kromosom, tetapi mempunyai jumlah kromosom dengan modal x=8 dan jumlah diploid 2n=4x=32. Akar yang diisolasi dari tanaman rumah kaca mempunyai

53,7% sel yang berjumlah kromosom normal diploid yaitu 2n = 32, dan 46,3% sel lainnya mempunyai kisaran jumlah kromosom dari 2n = 22 sampai dengan 2n = 64. Akar yang diisolasi dari planlet yang tumbuh di media MS padat hanya mempunyai 36,1% sel dengan jumlah kromosom normal 2n=32, sedangkan akar yang ditumbuhkan dalam media cair mempunyai 49,4% sel dengan jumlah 2n=32. Jumlah kromosom pada akar rambut bervariasi tergantung dari galur *Agrobacterium* yang dipergunakan untuk infeksi eksplan tanaman. Akar rambut hasil transformasi dengan galur bakteri 07-20001 mempunyai stabilitas kromosom yang paling tinggi dengan jumlah normal diploid 2n = 32 (62,4%), diikuti oleh akar rambut dari galur ATCC-15834 (61,9%), R1000 (43,6%) dan A4 (43,0%). Kisaran jumlah kromosom pada akar dari tanaman rumah kaca dan dari media MS padat dan cair adalah 2n=17-64, sedangkan kisaran jumlah kromosom akar rambut adalah 2n=11-66.

Kata kunci : kultur akar normal dan akar rambut, jumlah kromosom, stabilitas genetik, *Artemisia cina.*

INTRODUCTION

Variation in chromosome numbers is a common phenomenon in plant cell and tissue culture and increases with the age of the culture. For example, in *Vicia faba*, aneuploidy increases with the increasing age of cultured callus (Jha & Roy, 1982), and in almond, increasing age results in high level of polyploidy as well as aneuploidy (Mehra & Mehra, 1974). Similar results were observed in maize (McCoy & Phillips, 1982), *Nicotiana* species (Nuti Ronchi *et al.*, 1981), *Hordeum* (Orton, 1980) and protoplast-derived potato plants (Karp *et al.*, 1982). In wheat plants regenerated from cultured immature embryos, structural chromosome variation such as translocations was also identified during meiosis (Karp & Maddock, 1984).

Root culture is usually established in order to investigate the secondary metabolite produced in roots. Untransformed roots or hairy roots as a result of transformation with *Agrobacterium rhizogenes* have certain advantages compared to cell and suspension cultures. Root cultures of some species have greater level of genotypic and phenotypic stability than dedifferentiated cells so that problems with culture variation and loss of productivity are alleviated (Hamill *et al.*, 1987). In some species, bioactive compounds produced in hairy roots are much greater than that in cell suspension cultures (Sasaki *et al.*, 1998; Lee *et al.*, 1999; Saito *et al*, 2001) or leaves of plantlets (Zarate, 1999) and varied depending on the plant genotype (Ermayanti, 1999). Aird *et al.* (1988) examined hairy root cultures cytologically to assess their chromosome numbers. All hairy root cultures of some species examined had correct 2n diploid number of chromosomes in root tip cells. This contrasted with their observation that in suspension cells of *Nicotiana rustica* and *Beta vulgaris* the chromosome numbers were variable with both polyploids and aneuploids.

Artemisia cina is a medicinal plant that usually used as anthelmintics especially for children, antibacterial, antifungal and antitumor because it produces alkaloids, saponin, flavonoid dan polyphenols (Syamsuhidayat & Hutapea 1991; Tan *et al.*, 1998). Chromosome number of genus *Artemisia* is x = 8 or 9. *A. cina* has unique chromosome numbers with both modal number of x = 8 and x = 9. The normal plants of these species have chromosome numbers of diploid 2n = 2 x = 18 (from modal x = 9) or of polyploid 2n = 4x = 32 (from modal x =8) (Darlington & Wylie, 1956; dePadua *et al.*, 1999). The aim of this study was to assess the genetic stability of root cultures of *Artemisia cina* by assessment of chromosome numbers in both untransformed and transformed root cultures. The genetic information between transformed root lines resulted after transformation with some strains of *Agrobacterium* is needed for manipulating levels of secondary products in

root cultures and of identifying the genes involved.

MATERIALS AND METHODS

Roots collected from intact plants of *A. cina* grown in glasshouse (originated from Medicinal Plant Research Division, Research and Development Centre for Health, Tawangmangu, Central Java, Indonesia) and those isolated from plantlets resulted from shoot multiplications, grown in solid and liquid MS medium (Murashige and Skoog, 1962) without hormones were used as cytological analysis of untransformed root cultures. *A. rhizogenes* strains 07-20001, ATCC-15834, A4 and *A. tumefaciens* strain R1000 were used for transformation of *A. cina* after 2 days grown in LB (for *A. tumefaciens* strain R1000) or YMB (for *A. rhizogenes* strains A4, 07-20001, and ATCC-15834) medium. Sterile leaf blades on MS medium were inoculated by wounding explants with a scalpel which had been dipped into the bacterial broth, then the explants were incubated at 28°C in darkness. After being freed from the bacteria using 100-200 mg/l cefotaxime, transformed root tips were cut at 1-2 cm and cultured in MS liquid medium without additions of exogenous hormones.

Cytological analysis was conducted by squash method (Karp, 1991). Root tips of each treatment were collected and immersed in saturated paradichloro-benzene for 3 hours at room temperature followed by overnight fixation in glacial acetic acid : ethanol (1:3). Subsequently, root tips were hydrolyzed in 1 N HCl at room temperature for 5 mins then washed in water and stained with 2% aceto orcein in 45% acetic acid. Finally root tips were squashed in 45% acetic acid and examined under microscope. Cells at metaphase were analyzed for chromosome number and a maximum of 10 clear cells from each root tip were scored. Cells that were broken or overlapped with the neighbouring cells were not involved. More than 30 root tips of each treatments (untransformed and each line of transformed roots) were excised and more than 100 clear cells were examined for chromosome counts.

RESULTS AND DISCUSSION

The results indicate that the normal chromosome number of *A. cina* roots was polyploid number with 4x = 2n = 32. It showed that untransformed roots (roots of glasshouse plants, roots isolated from plantlets cultured in solid and liquid MS medium) had variation in chromosome numbers which ranged from 2n=17 to 2n=64. From 192 clear metaphase cells of glasshouse plants showed 53.7% of cells had correct chromosome numbers of 2n=32, 15.2% of cells had chromosome numbers lower than the normal 2n numbers and 31.3% of cells had higher than normal chromosome numbers (Table 1). The chromosome numbers of glasshouse plants ranged from 2n=22-64 (Fig.1). Only one cell out of 192 cells examined had polyploid numbers of 4n=64. None of the cells had haploid numbers.

Roots isolated from both plantlets grown in solid MS medium or from untransformed roots grown in liquid medium with no addition of exogenous hormones had greater variation of chromosome number compared to that of roots of glasshouse plants (Table 1). Roots in solid medium found to have chromosome numbers ranged from 2n=17 to 2n=62 (Fig.2), whilst roots in liquid culture had 2n=18-63 chromosomes (Fig.3). Roots grown in MS liquid medium was very slow so that the samples taken for chromosome analysis were less than 100 cells. Only few clear root tips which had clear metaphase cells found in this experiment. The lower genetic stability in cultures compared with that of plants grown *ex vitro* is a common phenomenon, many factors contributes to genetic instability of cultures including cell

Table1. Chromosome number of untransformed and transformed roots of *Artemisia cina*.

Source of roots	Number of cells with various chromosome number (2n)					Total cells observed
	11 (%)	16 (%)	17-31 (%)	32 (%)	33-66 (%)	
Glasshouse plants	0	0	29 (15.2)	103 (53.7)	60 (31.3)	192
Plantlets grown in MS solid medium	0	0	30 (24.6)	44 (36.1)	48 (39.3)	122
Roots grown in MS liquid medium	0	0	25 (31.7)	39 (49.4)	15 (18.9)	79
Transformed roots strain 07-20001		1 (0.5)	33 (16.1)	128 (62.4)	43 (21.0)	205
Transformed roots strain ATCC-15834	0	0	38 (19.5)	120 (61.9)	36 (18.6)	194
Transformed roots strain R1000	1 (1.0)	0	39 (38.6)	44 (43.6)	17 (16.8)	101
Transformed roots strain A4	0	0	61 (24.3)	108 (43.0)	82 (32.7)	251

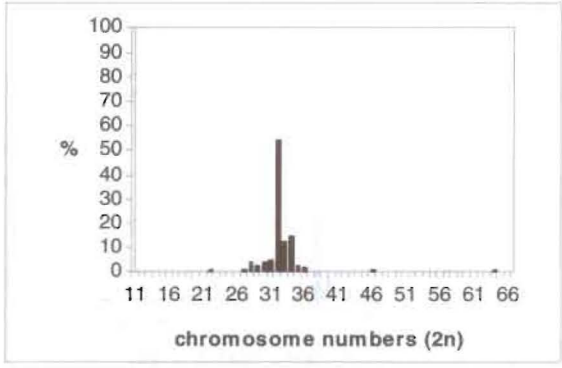

Figure 1. Cytological analysis of *A. cina* roots isolated from planlets grown in glasshouse.

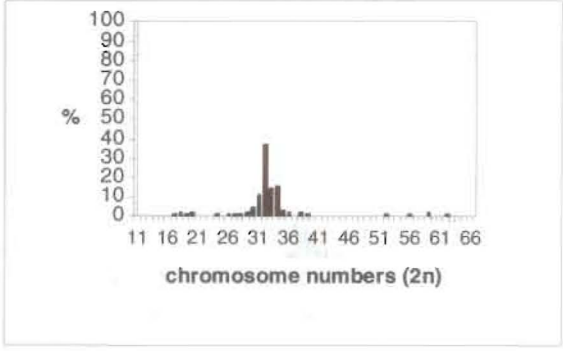

Figure 2. Cytological analysis of *A. cina* roots isolated from planlets cultured in MS solid medium without hormones.

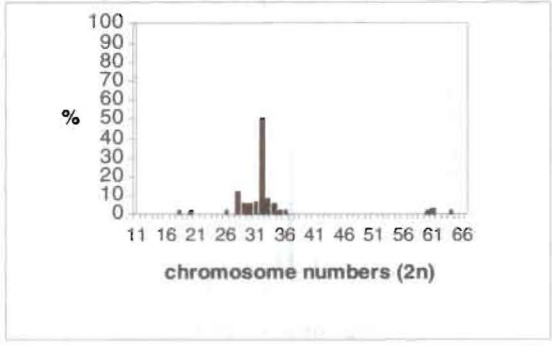

Figure 3. Cytological analysis of *A. cina* untransformed roots cultured in MS liquid medium without hormones.

aging, composition of mineral elements, environmental stress and DNA changes (D'Amato, 1990). These factors could occur in untransformed roots of *A. cina* observed in this experiment.

In transformed roots of *A. cina*, genetic stability was affected by strains of *Agrobacterium*. Roots transformed with *A. rhizogenes* strain 07-20001 was the most stable compared to those transformed with other strains of *Agrobacterium* (Table 1), with range of chromosome numbers from 2n=16 to 2n=43, and one out of 205 cells examined had chromosome number of 2n=66. One cell

had haploid chromosome number of 2n=16 which found in separate root culture (Fig.4). Chromosome numbers of roots transformed with *Agrobacterium* strain ATCC-15834 ranged from 2n=18 to 2n=60 (Fig.5) with 61.9% of them had normal diploid chromosome numbers of 2n-32, 19.5% of cells found to have chromosome numbers lower than the diploid numbers and 18.6% of cells had higher chromosome number than that of the diploid number. Roots transformed with *A. tumefaciens* strain R1000 had chromosome numbers ranged from 2n=17 to 2n=59 (Fig. 6). Roots transformed with *A. rhizogenes* strain A4 was the most unstable line. Only 43.0% of cells had correct number of chromosomes, 24.3% of cells had chromosome number lower than diploid, and 32.7% of cells had chromosome numbers higher than that of diploid numbers. The distribution of the chromosome numbers of roots transformed with *Agrobacterium* strain A4 is presented in Fig. 7.

The low genetic stability as showed by cytological analysis in *A. cina* transformed roots was in contrast with that of some other species. In *Swainsona galegifolia*, transformed root cultures resulted after transformation with *A. rhizogenes* strains LBA 9402 and A4 had more than 90% normal diploid chromosome numbers. This also the case of untransformed roots grown in some different medium and roots isolated from seedlings and germinated seeds. The results were in contrary with callus culture and roots regenerated from callus of *S. galegifolia* (Ermayanti *et al.*, 1993). Hairy roots of *A. annua* after infection *with A. rhizogenes* strain LBA-9402 had stable chromosome numbers

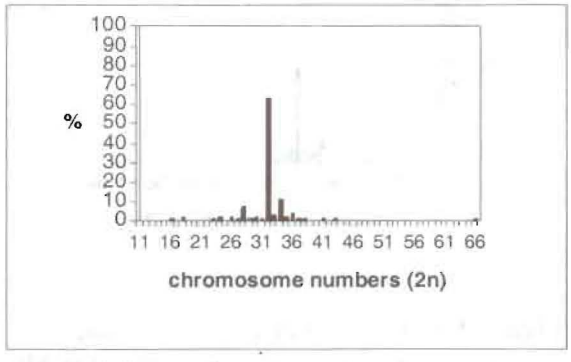

Figure 4. Cytological analysis of *A. cina* hairy roots transformed with *A. rhizogenes* 07-20001.

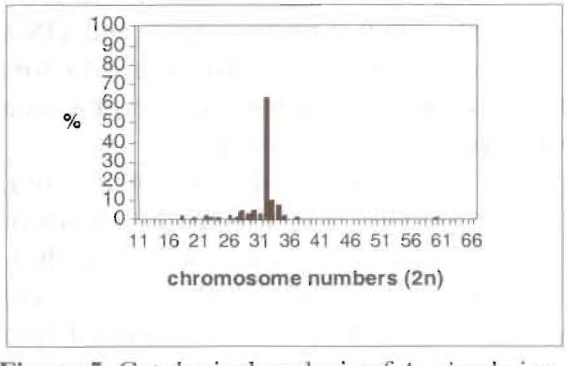

Figure 5. Cytological analysis of *A. cina* hairy roots transformed with *A. rhizogenes* ATCC-15834.

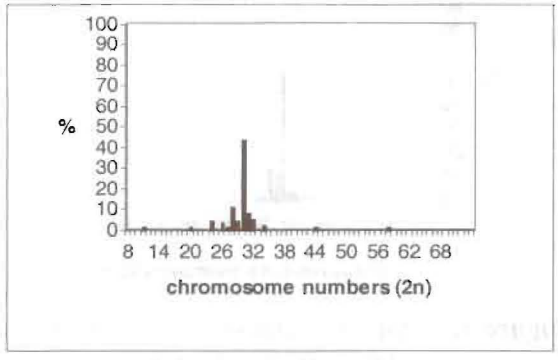

Figure 6. Cytological analysis of *A. cina* hairy roots transformed with *A. tumefaciens* R1000.

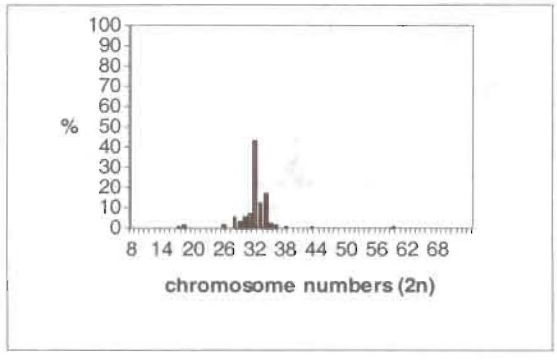

Figure 7. Cytological analysis of *A. cina* hairy roots transformed with *A. rhizogenes* A4.

of 2n = 18 after 20 months in culture medium (Mukherjee *et al.*, 1994).

Growth of transformed roots of *A. cina* in MS medium without hormones was higher than that of untransformed roots (Aryanti *et al.*, 2001). However, growth of hairy root lines resulted from transformation with *Agrobacterium* strains R1000 was lower than hairy roots transformed with *Agrobacterium* strains 07-20001, A4 and ATCC-15834. The root tips of hairy roots lines R1000 were very quickly turned brown. This problem, therefore, caused the difficulty to find clear metaphase cells for chromosome examination. Such problem was not found for hairy roots lines of *Agrobacterium* strains 07-20001, ATCC-15834 and A4, so that more clear metaphase cells could be examined for the chromosome analysis.

PCR analysis showed that only T_L region of T-DNA plasmid was detected (Aryanti *et al.*, 2001). The integration of the T-DNA which was only for T_L-DNA in *A. cina* may be related to the loss of some chromosomes in most root lines. This result is in contrast with *Atropa belladonna* transformed with *A. rhizogenes* strain ATCC-15834, both T_L- and T_R-DNA were integrated in the transformed roots (Aoki *et al.*, 1997). In *Azadiracta indica* roots, the transformation event affected the integration of the T-DNA to the plant genome. Both T_L- and T_R-DNA regions were detected in two out of three root clones examined, whilst one root clone only contained T_L-DNA region (Ermayanti *et al.*, 2000). The selection of root lines from each clone of *A. cina* could be investigated to obtain stable root clones which may result in the stable production of bioactive compounds of the roots.

The low stability in transformed roots of *A. cina* may be caused by structural rearrangements as that in *Vicia faba* that found that 50% of transformed roots examined had polyploid (2x-9x) and 6% had aneuploid chromosome numbers or structural rearrangements (Ramsay & Kumar, 1990). The chromosomes become eliminated apparently at random. In somatic hybrid cells, the chromosomes of one of the parents may be eliminated preferentially. This could be the case of *A. cina* roots. In hairy roots, cytological changes were also detected in transformed root cultures of *Trifolium pratense* and in regenerated plants of *Lotus corniculatus* (Webb *et al.*, 1990). The reduction of chromosome numbers and the loss of regeneration ability during subculture had occurred in hairy root cultures of *Onobrychis viciaefolia* (Xu & Jia, 1996). This may be the case for *A. cina*, that transformed roots used in this experiment had been subcultured for 3-6 times, the age of the cultures was about 6-12 months. To confirm the effect of culture age and subculture on genetic stability of *A. cina* roots, cytological examination of germinated seeds and primary roots grown directly at the wounded site of *Agrobacterium* infection should be done, however, seed germination of *A. cina* was very low. Instability of phenotype and gene expression in long-term culture also occurred in carrot hairy root clones (Guivarc'h *et al.*, 1999), in contrast with the hairy root clones of tomato which was stable for 50 subcultures over 25 months (Joao & Brown, 1994). Opine and PCR analysis should be done to confirm the gene expression in long-term culture of *A. cina*. The fact that untransformed and transformed roots of *A. cina* have unstable in chromosome numbers most probably is caused by the variation in chromosome numbers of the intact plants themselves (Table 1). Germinated seeds are needed to confirm this genetic variability. However, the ability of seed gemination was very low. None of seeds harvested from three plants grown in the field germinated either in mixture of soil with sand or in a culture medium.

The genus *Artemisia* is placed in the tribe of Anthemideae, consists of more than 200 species. The modal of chromosome number of genus *Artemisia* is x = 8 or 9. Some species of this genus including *A. cina* has

unique chromosome numbers with both modal number of x = 8 and x = 9. The normal plants of these species may have chromosome numbers of diploid 2n = 2 x = 18 (from modal x = 9) or of polyploid 2n = 4x = 32 (from modal x =8) (Darlington & Wylie, 1956; dePadua *et al.*, 1999). The differences in chromosome numbers could rely on the origin and the distribution. Most of the species are native of Eurasia and North America. These species are found in central and South-Western Asia which is thought to have been originated here, and to have immigrated to North America. Some species have been introduced in the Malesiana area, including Indonesia, usually as ornamental and medicinal plants (dePadua *et al.*, 1999). *A. cina* used in this experiment was originated from Tawangmangu (Central Java, Indonesia). There is no report on the variation of chromosome on *A. cina* grown in Indonesia. Therefore, further investigation on chromosome analysis of this plant grown in Indonesia is needed to confirm whether the variation in chromosome number is a common phenomenon as in the root cultures.

In conclusion, root cultures of *A. cina* has variation in chromosome numbers with basic diploid number of 2n=32. Variation in the chromosome number was also detected in roots of plants grown in the glasshouse. In transformed roots, roots transformed with *A. rhizogenes* strain 07-20001 was the most stable whilst roots transformed with *A. rhizogenes* strain A4 was the most unstable line. This information on variation in chromosome numbers may also contribute to the further investigation of root culture of *A. cina* or for the genetic manipulation.

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