HortScience 31(5):855–857. 1996.

A New Procedure to Prepare Slides of Metaphase Chromosomes of Roses

Yan Ma[1], M. Nurul Islam-Faridi[2], Charles F. Crane[3], David M. Stelly[4], H. James Price[4], and David H. Byrne[5]
Texas A&M University, College Station, TX 77843-2133

Additional index words. rose, shoot tip, cell suspension, chromosome, *Rosa*

Abstract. **To our knowledge, there has been no published technique to produce consistently high-quality slides of somatic chromosomes of roses (*Rosa* sp.). Therefore, various pretreatments, fixatives, digestions, stains, and maceration and squashing methods were tested to identify a procedure to produce clear, well-spread chromosomes from shoot tips. The best results were obtained after pretreatment in a mixture of 0.1% colchicine and 0.001 M 8-hydroxyquinoline for 4 h, and fixation in 2 acetone : 1 acetic acid (v/v) with 2% (w/v) polyvinylpyrrolidone. The darkest-stained chromosomes were obtained with carbolfuchsin staining of air-dried cell suspensions that had been spread in 3 ethanol : 1 acetic acid (v/v).**

Plant breeders and cytogeneticists have long valued the analysis of somatic metaphase chromosomes for elucidating genomic relationships. This task has been difficult with roses, since the chromosomes are small, the mitotic index is generally low in shoot tips, root development is weak, roots are thin in mature individuals, and most seed is difficult to germinate. Previous studies have reported chromosome numbers, but the chromosomes have not been sufficiently spread to permit detailed banding or karyotyping (Ma and Chen, 1991, 1992; Stebbins, 1971; Täckholm, 1922). Spreading the chromosomes has often required so much force that the chromosomes break (Hömmö and Särkilahti, 1986). Furthermore, conventionally squashed chromosomes are embedded in too much cytoplasm to permit robust in situ hybridization. Here we report an improved protocol using enzymatic digestion (Jewell and Islam-Faridi, 1994) and spreading of whole shoot tips or cell suspensions to prepare somatic metaphase chromosomes of roses.

Materials and Methods

The rose (genus *Rosa* L.) clones used to prepare the metaphase slides were the modern cultivars Angel Face and Sunflare, and the interspecific hybrid 89-1 (*Rosa wichuraiana* Crép x *R. roxburghii* Thratt.). All were grown in a space-planted test field. The following protocol was developed through multiple experiments in which individual steps were varied to search for improvements.

Collection of material

The most accessible, mitotically active material from established plants was the terminal 2 to 4 mm of a healthy, actively growing shoot collected during the springtime burst of growth. The mitotic index was highest when 10- to 15-mm shoot tips were collected in the morning and held in ice water (0 °C) for transport to the laboratory. Five to 10 shoot tips could be carried in one 5-mL tube, and specimens were generally put into the pretreatment within 60 min from collection. In the laboratory, the young outside leaves were carefully removed. The terminal 2- to 4-mm portion of the shoot apex was cut off and placed immediately in a pretreatment solution.

Pretreatment

Three pretreatment solutions were evaluated for their ability to spread the chromosomes and arrest the dividing cells at metaphase, thus increasing the number of divisions available for study. The solutions were 0.002 M 8-hydroxyquinoline, 0.1% colchicine, and 0.001 M 8-hydroxyquinoline combined with 0.1% colchicine. Each was tried for 2-, 3-, and 4-h incubations of shoot apices in the dark at ≈25 °C.

Fixation

Four fixative solutions were tested: 3 absolute ethanol : 1 acetic acid (v/v), glacial acetic acid, 3 dimethyl sulfoxide : 1 chloroform (v/v), and 2 acetone : 1 acetic acid (v/v) + 2% (w/v) polyvinylpyrrolidone (PVP) of molecular weight 40,000. All fixatives were freshly prepared, and the tissue was fixed for <24 h at ambient pressure.

Washing

After fixation, the shoot tips were soaked in distilled water for 1 to 24 h to elute the fixative and soften the tissue. If the shoot tips were fixed for 2 to 4 h at 25 °C, they became sufficiently soft upon overnight refrigeration in distilled water.

Acid digestion

The shoot tips were exposed to combinations of three HCl concentrations (0.5, 1.0, and 2.0 N) and three durations (15, 20, and 30 min) at 25 °C.

Equilibration

After being rinsed twice in distilled water and soaking for 10 min in distilled water, the shoot tips were incubated in 0.075 M KCl for 30 or 60 min or, alternatively, in 0.01 M sodium citrate at pH 4.6 for 15, 30, or 60 min at ≈25 °C.

Enzymatic digestion

Equilibrated shoot tips were incubated in 20 to 50 µL (twice to thrice their own volume) of cell-wall lytic enzymes in 0.5-mL microcentrifuge tubes. Optimum digestion was obtained with 5.5% cellulase R10 (Yakult Honsha Pharmaceutical, Tokyo) and 1.25% pectolyase Y23 (Seishin, Tokyo) in 0.01 M sodium citrate at pH 4.6. The enzymatic digestion ran 1, 2, 3, or 4 h at 37 °C.

Slide preparation

There were two procedures to prepare slides. In the first, digested shoot tips were macerated and spread on the slide; in the second, shoot tips were first dispersed into a protoplast suspension that was pipetted onto slides.

On-slide maceration. Individual shoot tips were placed on a microscope slide and blotted free of excess water. Two to three drops of 3:1 or 1 ethanol : 1 acetic acid fixative were added, and the tissue was gently dispersed with forcep tips. The uncovered preparation was examined briefly at ×160, until most cells had settled onto the surface of the slide. Then cytoplasmic debris was washed off with additional drops of 3:1 or 1 ethanol : 1 acetic acid, and excess liquid was absorbed from the edges of the slide with filter paper. The slide was air-dried, stained, and covered with a cover slip.

Suspension and spreading of protoplasts. About 480 µL of distilled water was added to the microcentrifuge tube containing the shoot tips and enzyme mix, and the tube was vigorously vortex-mixed 30 to 60 s to break up the shoot tips. Persistently undissociated pieces were ignored at this stage and discarded after the next stage, since the mitotic cells themselves were relatively fragile. The suspension of fixed protoplasts was centrifuged at ≈700 g_n for 5 min at 2 to 4 °C, and the pellet was resuspended in ≈500 µL of distilled water. The larger pieces of debris were allowed to settle out for a few minutes, and then the supernatant

Received for publication 23 Oct. 1995. Accepted for publication 26 Mar. 1996. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

[1]Postdoctoral Research Associate, Dept. of Horticultural Sciences.
[2]Assistant Research Scientist, Dept. of Soil and Crop Sciences.
[3]Research Scientist, Dept. of Soil and Crop Sciences.
[4]Professor, Dept. of Soil and Crop Sciences.
[5]Associate Professor, Dept. of Horticultural Science. To whom reprint requests should be addressed.

cell suspension was moved to a fresh microcentrifuge tube. The suspension was again centrifuged at ≈700 g_n for 5 min at 2 to 4 °C and resuspended in ≈500 µL of 3 ethanol : 1 acetic acid. The suspension was allowed to settle briefly and then moved to a fresh microcentrifuge tube. After centrifugation at ≈700 g_n for 5 min at 2 to 4 °C, the pellet was resuspended in 20 to 40 µL of 3 ethanol : 1 acetic acid per shoot tip, and the microcentrifuge tube was placed on ice until used. The suspension was mixed by hand-tapping and immediately spread by pipetting 5 to 10 µL of it onto a scrupulously clean microscope slide that had been washed with 95% ethanol and wiped dry. The suspension was allowed to spread out and air-dry without further disturbance. A drop of stain and a cover slip were applied to the dried slide, which was then heated to 87 °C for ≈20 s and squashed on a hot plate at 87 °C.

Table 1. Effect of chemical pretreatments in darkness at 25 °C on yield and contraction of metaphase chromosomes from rose shoot tips.

Pretreatments	Duration (h)	Effect[z]
0.001 M 8-hydroxyquinoline with 0.1% colchicine	2	+
	3	++
	4	+++
0.002 M 8-hydroxyquinoline	2	+
	3	+
	4	++
0.1% colchicine	2	+
	3	++
	4	+

[z]+, ++, and +++ denote increasing contraction and yield of metaphase chromosomes.

Table 2. The effect of fixatives applied for 2 to 24 h on chromosome spreading from shoot tips.

Fixatives	Temp (°C)	Result[z]
3 Ethanol : 1 acetic acid (v/v)	25	+
Glacial acetic acid	4	++
3 Dimethyl sulfoxide : 1 chloroform (v/v)	25	++
2 Acetone : 1 acetic acid (v/v) + 2% PVP (w/v)	25	+++

[z]+, ++, and +++ denote increasing chromosome spreading in softer cytoplasm.

Stains

Carbol-fuchsin (Li and Zhang, 1991), acetocarmine (1% carmine in 45% acetic acid), and Giemsa stain (diluted 1:20 with distilled water) were tried.

Results and Discussion

The highest yield of divisions with distinct, fully metaphasic chromosomes followed a 4-h pretreatment with a solution that was 0.001 M with respect to 8-hydroxyquinoline and 0.1% with respect to colchicine in darkness at ≈25 °C (Table 1). With colchicine alone, the chromosomes clumped excessively, even when dividing cells could be obtained, and the centromere often was not clearly visible as a primary constriction. With 8-hydroxyquinoline alone, there were few metaphase divisions, and prophases were relatively more abundant. Colchicine depolymerized microtubules (Eigsti, 1957), while 8-hydroxyquinoline apparently slowed the rate of progression among mitotic stages and impaired chromosome movement. Thus, both in combination improved chromosome spreading and contraction over either alone (Li and Zhang, 1991).

The tissue was hardest after the 3 ethanol : 1 acetic acid fixative solution (Table 2). The best chromosome morphology and spreading were obtained after 2 acetone : 1 acetic acid plus 2% PVP. The PVP bound the tannins and other phenolics that otherwise would have reacted with cytoplasmic proteins. Consequently, the cytoplasm was softer after fixation in solutions that contained PVP.

The best combination of chromosome spreading and distinct morphology was obtained after 20 min in 1.0 N HCl at ≈25 °C. Chromosome structure was degraded to various degrees after stronger HCl treatments, including 1.0 N HCl for 30 min or 2.0 N HCl for 15 min. The chromosomes were destroyed in 2.0 N HCl at 20 or 30 min. Hydrochloric acid attacks chromosomes in several ways. It extracts histones and other basic proteins from the chromatin, denatures DNA, and at sufficient concentration depurinates DNA. Thus, the HCl treatment must be carefully controlled to be beneficial. Sodium citrate at pH 4.6 was the best equilibrating buffer before enzymatic digestion, but duration of treatment mattered little between 15 and 60 min. The chromosomes spread the most after 3 to 4 h of enzymatic digestion, which is far longer than is needed in corn (*Zea mays* L.) or cotton (*Gossypium hirsutum* L.) (Hanson et al., 1995; Jewell and Islam-Faridi, 1994).

Among the tested stains, carbol-fuchsin stained the chromosomes most intensely and differentially, and the stain intensified with time. Giemsa stain stained the chromosomes weakly, with little contrast from the stained cytoplasm, and left particulate debris across the specimen. Chromosomes stained in acetocarmine faded with time and lacked sufficient contrast for good photography, even though the same batch of stain has been routinely used to stain cotton chromosomes. Acetocarmine requires iron for effective chromosome staining; carbol-fuchsin does not. The fainter staining with acetocarmine might have reflected inadequate iron in the specimen.

In the procedure to spread individual shoot tips on slides, the chromosomes spread more in 1:1 than in 3 ethanol : 1 acetic acid. Although either preparation method (Figs. 1–3) can yield chromosome spreading, exposure, and morphology that suffice for in situ DNA hybridization, the suspension method requires less skill to remove cytoplasmic debris without stripping the cells from the slide than does on-slide maceration. The suspension method also allows the shoot tips to be digested more completely before spreading, as the digested shoot tip need not be intact during handling. In our experience, the suspension method yields flatter, more uniformly clean spreads than does the on-slide maceration.

In concept, this spreading procedure is applicable to shoot or root tips from any species of vascular plant. It can be summarized as follows:

1) Fix the pretreated shoot or root tips 12 to 28 h at room temperature in an appropriate fixative, e.g., 3 ethanol : 1 acetic acid for most plants. Use PVP in the fixative if phenolics are a problem.

2) Wash the shoot or root tips in several changes of reagent-grade water. Incubate them in the last change for ≈30 min to 2 h, depending on the root or shoot thickness.

3) Treat the tissue in dilute HCl for an appropriate interval at ≈ 25 °C. The optimum concentration and duration depend on the species; 1 N HCl for 20 min works for roses, but 0.1 N HCl for 5 to 10 min is more nearly optimal for most species.

4) Wash out the HCl with reagent-grade water.

5) Equilibrate the tips with 0.01 M sodium citrate, pH 4.6, for 5 to 30 min.

6) Digest the tips in 5% cellulase, 1% pectolyase in 0.01 M sodium citrate at pH 4.6. Use 20 to 40 µL of enzyme mix per tip. The optimum interval in roses is 3 to 4 h, but in most species this seriously degrades chromosome morphology. The optimum digestion interval depends also on the size of the tips, but after acid treatment, it is from 15 to 90 min for most species.

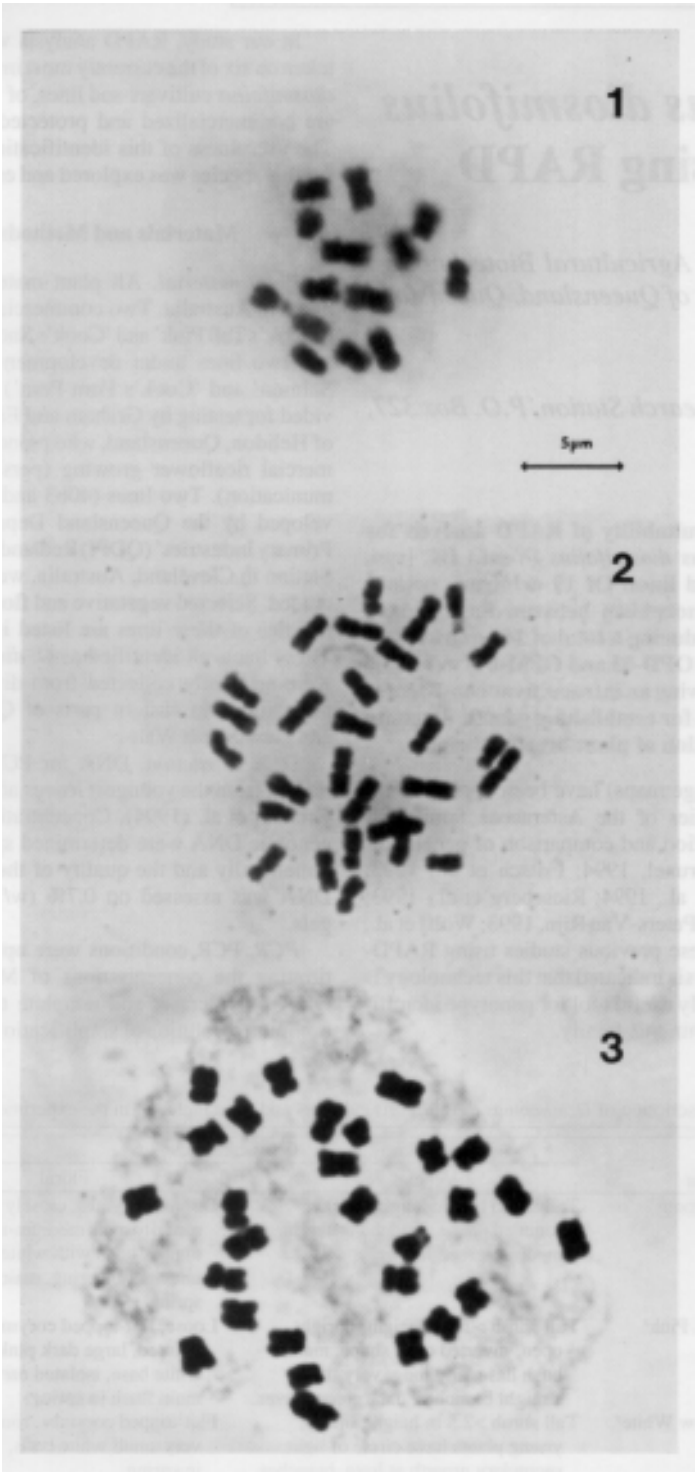
7) At the end of digestion, fill the microcentrifuge tube with reagent-grade water and briefly vortex-mix it to disperse the cells. Centrifuge the contents at 700 g_n for 5 min at 2 to 4 °C and discard the supernatant.

8) Resuspend the pellet in reagent-grade water. If there are large, undissociated pieces, allow them to settle out for a few minutes and transfer the supernatant to a new microcentrifuge tube.

9) Centrifuge the suspension at 700 g_n for 5 min at 2 to 4 °C, discard the supernatant, and resuspend the pellet in freshly made 3 ethanol : 1 acetic acid.

10) Centrifuge the suspension at 700 g_n for 5 min at 2 to 4 °C, discard the supernatant, and resuspend the pellet in 15 to 20 µL freshly made 3 ethanol : 1 acetic acid per tip.

11) Apply 5 to 10 µL of the protoplast suspension to a scrupulously clean microscope slide and allow it to air-dry without disturbance at ≈30% to 50% relative humidity.



Figs. 1–3. (**1**) Cell from hybrid genotype 89-1 (*Rosa wichuraiana* Crép x *R. roxburghii* Thratt.) prepared using the on-slide maceration protocol ($2n = 14$). This and the remaining pictured cells were stained in carbol-fuchsin. (**2**) Cell from 'Sunflare' rose prepared using the suspension protocol ($2n = 4x = 28$). (**3**) Cell from 'Angel Face' rose prepared using the suspension protocol ($2n = 4x = 28$).

To clean the slides beforehand, scrub them in a 9:1 (v/v) mixture of 95% ethanol and glacial acetic acid, and wipe them with fresh, oil-free tissue paper. The suspension must spread rapidly and evenly in all directions when it is applied to the slides, or else the cells will tend to burst and lose chromosomes.

12) If the slide is to be used for in situ DNA hybridization, allow it to air-dry for 2 to 3 days at low humidity to improve cell retention.

Literature Cited

Eigsti, O.J. 1957. Colchicine—In agriculture, medicine, biology, and chemistry. Iowa State College Press, Ames.

Hanson, R.E., M.S. Zwick, S. Choi, M.N. Islam-Faridi, T.D. McKnight, R.A. Wing, H.J. Price, and D.M. Stelly. 1995. Fluorescent in situ hybridization of a bacterial artificial chromosome. Genome 38(4):646–651.

Hömmö, L.M. and E.M. Särkilahti. 1986. A method of counting chromosomes of hardwood trees using root tips and young leaves. Can. J. For. Res. 16(2):401–403.

Jewell, G.D. and N. Islam-Faridi. 1994. A technique for somatic chromosome preparation and C-banding of maize, p. 484–493. In: M. Freeling and V. Walbot (eds.). The maize handbook. Springer-Verlag, New York.

Li, M.-X. and X.-F. Zhang. 1991. Techniques for plant chromosome research. Northeast For. Univ., Harbin, China.

Ma, Y. and J.-Y. Chen. 1991. Chromosome studies of 7 roses. J. Fujian College of For. 11(2):215–218.

Ma, Y. and J.-Y. Chen. 1992. Chromosome studies of 6 species of *Rosa* in China. Guihaia 12(4):333–336.

Stebbins, G.L. 1971. Chromosomal evolution in higher plants. Edward Arnold, London.

Täckholm, G. 1922. Zytologische Studien über die Gattung *Rosa*. Acta Hort. Bergiani 7, no. 3:98–380.