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Optimization of Giemsa staining protocol for metaphase chromosome preparation using leaf bud meristem of *Garcinia indica* (Thouars) Choisy for karyotype analysis

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ABSTRACT

Garcinia indica is a polygamodioecious tree. Being woody plant chromosome count is often limited by their small and huge chromosome number. In any plant obtaining well-spread and less distorted chromosome preparations by conventional squash spread method is intricate due to the presence of a cell wall. The present study described in detailed the method of enzyme digestion mediated protoplast dropping for preparing metaphase chromosomes from plant leaf tissues. Pretreatment of samples with 2 mM 8- hydroxyquinoline solution was seen to be best to accumulate the maximum (Mitotic index: 71.4%) Number of metaphases. The best chromosomal morphology and spreading were obtained after methanol: Glacial acetic acid (3:1) fixative treatment with clear cytoplasmic background. Among the various concentrations of cellulase/pectinase used in the study, a maximum number of protoplast were released after 3 hours treatment with 5 % cellulase/pectinase mixture. Chromosome staining with giemsa stain for 10 min was observed to be sufficient to stain the chromosomes more intensely with cleared morphological details. The methodology described here offers a reliable method for chromosome preparation and is suitable for routine karyotype analysis of large sample sizes.

Keywords: *Garcinia indicia, Cellulase, Protoplast, Chromosome, Giemsa, Karyotype*

INTRODUCTION

karyotyping and chromosome count provide essential information for the study of plant evolution. Since chromosome number analysis is the quickest, least expensive, and most practical way to learn in-depth information about the genome of a specific species, it is of interest to many cytotaxonomists [9]. A crucial step in all cytogenetic techniques is chromosome preparation. The majority of contemporary molecular cytogenetic methods, including Tyramide-FISH, GISH, and FISH, require evenly distributed and morphologically intact chromosomes [14b].

Plant chromosome preparation is particularly difficult because of the species-specific variations in cytoplasm consistency and the presence of rigid cell walls [1]. The most popular traditional method of preparing plant chromosomes, the "squash spread technique," has been questioning in several studies [3, 5, 11]. The squash technique is thought to require expertise and skillful work but yields a relatively low fraction good spread [20]. Therefore, we have standardized anenzyme mediated protoplast-dropping technique for *Garcinia indica* in the current study as it produces a large number of flat, evenly distributed preparations with good chromosomal morphology that are free of cytoplasmic remnants and other cellular material.

Garcinia indica Choisy (family Clusiaceae) is a

polygamodioecious tree also known as 'kokum', is endemic to the Western Ghats of India. It is a well-known Indian spice tree that is frequently used in the preparation of curries and has many industrial and medicinal uses. Being polygamodioecious, plants exhibit considerable variation in their height, branching structure, canopy, and leaf and fruit morphology [4].

To determine if the polymorphism occurred at the genetic level, earlier karyomorphological investigations of G. indica were done. The studies indicated chromosomal numbers 2n = 54 [5] and 2n = 48 [27], respectively. Nevertheless, the chromosomal numbers in both investigations were different, thus a recent karyomorphological experiment was redone using the root tip of *G. indica* seedlings by squash method, and the study validated chromosome number 2n = 54 [4].

But for confirmation of chromosome number and for other population-related studies in plants, seedling root tips can't be the right choice for the isolation of chromosomes. As cytogenetics cannot be used in population studies unless samples are taken from real plants in the field[3]. Hence in the present study, we have used leaf bud meristem tissue for chromosome isolation and standardized enzyme-mediated protoplast dropping protocol for it. The protocol described here is modified from the shoot tip enzyme digestion protocols of Birch tree [3]and Rose plant [19] and it is developed after multiple experimental trials at every step.

MATERIALS AND METHODS

Chemicals and reagents

Pretreatment chemicals: Saturated solution of PDB (Paradichlorobenzene) and 2 mM 8-hydroxyquinoline solution was used to arrest metaphases in freshly collected leaf buds.

Fixative: Mixture of 1 part of glacial acetic acid and 3 parts of absolute ethanol/methanol, prepared fresh every time.

Digestive enzyme

1. Cellulase stock: 10 % cellulase (w/v) in 50 mM acetate buffer of pH 4.8 containing 75 mM KCl and 7.5 mM EDTA.

2. Pectinase stock: 10% pectinase (w/v) in 50 mM acetate buffer of pH 5.4 containing 75 mM KCl and 7.5 mM EDTA. Both the enzyme stocks store at -20 °C temperature.

Chromic acid: Chromic acid is used for cleaning microscopic slides before use.

Giemsa stain: 2% giemsa stain diluted with 12.5 mM Sorenson's Buffer (pH 6.8) was used to stain the chromosome spread.

Collection of plant material

Leaf buds of size 1 cm – 2 cm of *G. indica* were collected in the morning at different time duration (9:30 am,10:00 am, 10:15 am,10:30 am, 10:45 am, 11:00 am, and 11:15 am) from medicinal and aromatic plants collection garden, KET's V. G. Vaze college Mulund(E), Mumbai. The collection of samples was done in October to December when the average temperature was in the range of 27 °C to 20 °C. The samples were immediately immersed in a pretreatment solution to arrest the metaphase followed by Fixation. For transport to the laboratory, fixed samples were placed in ice water (0 °C). These materials were maintained in the laboratory in the refrigerator until chromosomal preparation could commence.

Pretreatment

Two pretreatment solutions were tested for their potential to spread chromosomes and stop dividing cells at metaphase. 2mM 8-hydroxyquinoline and PDB were used in the experiments and each was tested for 2, 3, and 4 hours respectively. Samples treated with 2mM 8-hydroxyquinoline were incubated in dark at 16 -18 °C. while samples in PDB were refrigerated at 4 °C.

Fixation

Two fixative solutions viz., absolute ethanol: Glacial acetic acid (3:1) and methanol: Glacial acetic acid (3:1) were tested. A sample stored in a fixative was placed in a refrigerator at 4 °C until the study. Every time freshly prepared fixative was used.

Washing

The Fixed leaves were placed in distilled water for 30 min at room temperature to elute the fixative and

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soften the tissues.

Acid digestion

The leaves were subjected to 1N HCl treatment for three times duration (5,10 and 15 minutes) at room temperature.

Equilibration

After acid digestion leaves samples were soaked in distilled water for 15 minutes, followed by incubation in 50 mM acetate buffer (1000 µl) of pH 4.8 for 15 minutes at room temperature.

Enzymatic digestion

Cellulase, a gift from Advance enzyme Sun Magnetica, India, and Pectinase (Himedia) were the cell wall lytic enzymes used in the present study. In 50 mM Acetate buffer of pH 4.8 and pH 5.4, different concentrations of cellulase (w/v) (1%, 2%, 3%, 4%, or 5%) and pectinase (w/v) (1%, 2%, 3%, 4% or 5%) were prepared respectively. Tip of Equilibrated leaf samples was removed and cut into small pieces which were placed in 100 µl of cellulase\ pectinase enzyme mixture (1:1) in 1500 µl microcentrifuge tube for different incubation periods (2h, 3h, 4h or 5h) at room temperature.

Protoplast isolation

To break the leaf tissues in its enzyme mixture about 900 µl of distilled water was added to the microcentrifuge tube and the tube was vigorously vortex-mixed for 30 to 60 s. Visible cell debris were removed manually and the mixture was spun down using a microfuge at 5000 rpm for 5min at 25 °C. The supernatant was discarded, 1000 µl of ice-cold fixative was added to the protoplast pellet and the pellet was spun down again at 5000 rpm for 5min and then the fixative was discarded. Fixative treatment was repeated twice or thrice by spinning down the protoplast pellet to get cleaned and fixed protoplast. Finally, the protoplast pellet was resuspended into 1000 µl fresh ice-cold fixative and preserved in refrigerator until used.

Protoplast dropping

Before slide preparation preserved protoplast suspension was spun down to get the pellet and the pellet was resuspended into 100 µl of fresh ice-cold fixative. The suspension in a microcentrifuge tube mixed by hand taping. About 10 µl -20 µl of protoplast suspension were taken into the Pasteur pipet and dropped down onto a scrupulously clean microscopic ice-cold slide from different (2',3', 4' or 5.2') heights. Dropped slides were immediately kept on hot plate for 15 min adjusted at 87 °C temperature.

Staining

Completely dried slides were immersed into a copulin jar containing 2% Giemsa diluted with 12.5 mM Sorenson's buffer (pH 6.8) for 10 minutes

Establishment of Ideogram

Metaphases with well-spread chromosomes were chosen and photographed using Olympus CX33 LED illuminated trinocular microscope, fitted with Magcam DC 5 camera, computer, and MagVision software.To determine the diploid chromosome number, microphotographs of at least 40 metaphases were examined. A cytogenetic tool, KaryoType software version 2.0, was used to measure the length of the chromosome and to construct an ideogram of the chromosome type[2]. Chromosome measurements were carried out on magnified microphotographs (6,500X).The terminology used to categorize chromosome were according to the standardized nomenclature [18].

RESULTS AND DISCUSSION

In the present investigation, an attempt has been made to standardize the metaphase spread protocol by the protoplast dropping method over the conventional squash spread technique. For the present experiment we have finalized young leaf tissues which are easily available throughout the year It is reported that young leaf tissues are a reliable source of metaphases in any plant species [3]. In the squash spread technique, the majority of the time, chromosomes either stick together or some are lost or float away during tapping and squashing. Additionally, when preparing one slide, it takes a lot of time and skill to neatly separate chromosomes from one another so that they can be quantified easily. At a time only one slide can be prepared from a single meristematic tissue by the squash spread technique. By the protoplast dropping, method chromosomes are spread apart at place free of cytoplasm and frequently less distorted than in squash preparation. Protoplast dropping is also convenient for the processing of large sample sizes all

Table 1. Effect of PDB and 8- hydroxyquinoline treatment on mitotic index at of different time (2h, 3h, or 4h) durations

Pretreatment	Mitotic index (%)		
	2h	3h	4h
PDB	-	-	65.5
8-hydroxyquinoline	-	71.4	69.8

Table 2. Mitotic index (MI) values at different time duration after pretreatment with 2mM 8-hydroxyquinoline

MI after 3h pretreatment with 8-hydroxyquinoline	Time slots (AM)							
	9:30	9:45	10:00	10:15	10:30	10:45	11:00	11:15
MI (%)	16.6	21.42	29.41	64.1	36.4	33.3	25	15.1

at a time.

Collection of plant material

For the present study, leaf buds of size 1cm – 2 cm of *G. indica* were plucked in the morning at different time duration (9:30, 9:45, 10:00, 10:15,10:30, 10:45, 11:00, 11:15 am) from medicinal and aromatic plants collection garden, KET's V. G. Vaze college Mulund(E), Mumbai. The collection of samples were done in October to December when the average temperature was in the range of 27 °C to 20 °C. During the standardization of protocol, different factors were taken into consideration to accumulate a large number of proper metaphases to facilitate the observation. One of them was the temperature, as temperature has a profound effect on cell mitosis.

Pretreatment

Pretreatment facilitates the accumulation of metaphases by inhibiting the formation of microtubules. This arrests the dividing cells at metaphase. Thus, the number of cells in metaphase increases. Further due to pretreatment, the chromosomes get more contracted and shorter, making chromosome count easier. Hence pretreatment is a crucial step in the chromosome analysis of any plant sample. It helps to obtain cytological preparations with high frequency of metaphases with clear chromosomal morphology. Nature and duration of pretreatment have significant effect establishment of the karyotype of a species. Several pretreatment agents have been described in a plant chromosome study. The most used agents are colchicine, 8-hydroxyquinoline, α–bromonaphthalene, p–dichlorobenzene (PDB), and cold water [8, 25].

However, 8- hydroxyquinoline and p–dichlorobenzene was found to best to arrest a large number of metaphase cells in plants like Soybean which have small size chromosomes [29]. Much earlier paradichlorobenzene was used for the same plant[23]. As in *G. indica* chromosomes are small in size we have shortlisted 2mM 8-hydroxyquinoline and saturated solution of PDB to arrest a maximum number of metaphase cells. In a recent publication on karyomorphological research in *G. indica*, it was noted that maximal dividing cells were arrested at metaphase in the root tips of seedlings of this plant by using a saturated solution of p-dichlorobenzene [4].
The samples collected were immediately transferred in 2mM 8 – hydroxyquinoline solution kept at 16 ^{0}C and in PDB solution kept at 4 ^{0}C for different time durations (2 hours, 3 hours, and 4 hours) and their effect on several metaphase cells, as well as the chromosomal contraction, was studied and data shown in Table 1. It was seen that PDB takes 4 hours

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to arrest the cells in metaphase (MI: 65.5%, Table 1), still, there were few metaphase cell divisions with the primary constriction not visible. At 2 hours and 3 hours, PDB treatment prophases were relatively more abundant.

In the case of 4 hours 2mM 8- hydroxyquinoline treatment number of metaphase arrested cells (MI: 69.8 %, Table 1) are more or less similar to that of metaphase cells obtained (Mitotic index (MI): 71.4%, Table 1) in 3 hours of 2mM 8- hydroxyquinoline treatment, hence; in the present work 3 hours 2mM 8- hydroxyquinoline treatment was shortlisted. Also, with the aid of this treatment, the cell population also tends to enable primary and secondary constriction and cytoplasm very clear.

8- hydroxyquinoline slow down the rate of progression among the mitotic stages and impaires chromosome movement [19]. For shoot tip pretreatment in rose plants, 0.002 M 8-hydroxyquinoline, 0.1% colchicine, or a combination of 0.001 M 8-hydroxyquinoline and 0.1% colchicine were utilised with varying incubation times of 2, 3, or 4 hours in the dark at 25 °C. The result indicated that among all the pretreatment, an increased number of metaphase cells were obtained when shoot tips were treated with the combination of 0.001 M 8-hydroxyquinoline and 0.1% colchicine for 4 hours in the dark at 25 °C [19]. while working with the root tips of 11 species and 7 chemotypes of Cymbopogon, authors used either p-dichlorobenzene, 8-hydroxyquinoline, or their mixture with 3-5 hours of pretreatment at 12-14 °C. The finding demonstrated 3 -7 well-scattered and properly contracted metaphase plates were obtained in different species[17]. The shoot tips of *Hibiscus rosa-sinensis* were pretreated using cold water, p-dichlorobenzene (P), 8-hydroxyquinoline (Q), and combinations of both agents. Each pretreatment was carried out for 3, 6, 12, and 14 hours and the results showed that different pretreatments have their optimum condition for accumulating cells in late prophase and metaphase [25].

Effect of time of prefixation on mitotic activity

A mitotic index is a crucial tool for tracking a cell's progress through the cell cycle, and the frequencies of the cells during the various mitotic phases also show the kinetics of the cell cycle [16, 24]. In the present work, leaf buds were excised at different periods (9:30, 9:45, 10:00, 10:15, 10:30, 10:45, 11:00, and 11:15 am) and they were treated with 2mM 8- hydroxyquinoline for 3 hours at 16 °C temperature and data recorded in Table 2. Among all the different periods, at 9:30 am and 9:45 am number of non-dividing cells and prophases was maximum hence MI was 16.6% and 21.42 % respectively. As time progressed from 10:00 am to 10: 30 am number of non-dividing cells decreased and the number of prophases and prometaphases were increased but still there very were few cells in metaphase. The mitotic index at 10:00 am, 10:15 am and 10:30 am was 29.41, 33.3, 36.4 % respectively. The highest (64.1%) mitotic index (Table 2) with distinct, fully metaphase chromosomes were reported at 10:45 am. As time advanced, at 11:00 am many of the cells were in metaphases but still numerous cells were in anaphase and telophase. Therefore, MI at 11:00 was 53.7%. At 11:15 am maximum cells were in anaphase and telophase, so the MI was 15.1 %. Therefore 10:45 am time slot was selected as an ideal period for the collection of shoot tips.

Fixative treatment

Suitable fixative is an essential requirement for studying chromosome structure as this step significantly influences the clarity of chromosome morphology. And minute details like primary and secondary constrictions become more pronounced [28]. In the present study two fixatives have been tried viz., absolute ethanol: Glacial acetic acid (3:1) or Methanol: Glacial acetic acid (3:1). When Absolute ethanol: Glacial acetic acid (3:1) was used as fixative, chromosomes were very close to each other. The chromosome spread showed cytoplasmic background due to which chromosomal morphology remained uncleared. The best chromosomal morphology and spreading were obtained after methanol: Glacial acetic acid (3:1) fixative treatment with the clear cytoplasmic background.

Ideally, fixative used in the study should penetrate quickly, not shrink the cytoplasm, and spread the chromosomes for counting, So that the counting of the chromosome becomes easier. Carnoy's solution is highly recommended fixative by cytologists as acetic acid in it precipitates nucleus protein and cytoplasm is precipitated by alcohol. The presence of acetic acid helps to some extent to prevent further alcohol-induced shrinkage and hardening of the cytoplasm [28]as, hardening of cytoplasm makes it more difficult to produce well-flattened squashes. Therefore it is recommended to use a fixative which is a mixture of low water content and rapidly penetrating components like a Cornoy's solution.

In *Coccinia grandis* (L.) Voigt, methanol: Glacial acetic acid (3:1) was used as a fixative to fix the pretreated root tips [6]. Whie, in Rose plant authors have observed that after treating shoot tips with ethanol: glacial acetic acid (3:1) fixative its tissue became hard to process further so they replaced it with acetone: acetic acid (2:1) fixative, which gave them a best chromosomal morphology and spreading [19]. The "steam Drop" technique was created employing root meristematic cells from 13 monocot and 7 dicot genera in order to establish a straightforward, dependable process for plant mitotic and meiotic chromosome production. When authors dropped cell suspension in ethanol: acetic acid (3:1) fixative on the slide, after evaporation they noted that in most metaphases the chromosomes remained closed together whereas they get good chromosome spreading when they dropped suspension in 96 % ethanol using 'steam Drop' method on the slide followed by addition of ethanol: acetic acid (3:1) fixative drop after complete evaporation of 96 % ethanol [13a].

Acid digestion and equilibration

During acid digestion, chromosomes are attacked in a variety of ways by hydrochloric acid. It removes histones and other basic proteins from chromatin, denatures DNA, and depurinates DNA at high enough concentrations. For the HCl treatment to be beneficial, it must be closely monitored [19]. In the present work best chromosomal spreading with distinct chromosomal morphology was reported with 10 minutes 1 N HCl treatment. Chromosome structure got degraded after 15 minutes of 1 N HCl treatment whereas the tissue Was not properly hydrolysed with 5 minutes of 1 N HCl treatment. A wash of 50 mM acetate buffer of pH 4.8 for 15 minutes was best to equilibrate the samples before enzymatic digestion.

Enzymatic digestion and protoplast formation

Recent developments in the techniques of culturing plant cells and protoplast isolation have opened the way for chromosome isolation in plants [10]. The squash spread technique, widely used in plant chromosome studies has been limited by the presence of rigid cell walls. The obstacles are obtaining flat fields, good spreads, and good staining of chromosomes required particularly in karyological analyses of somatic tissues. However, protoplast technology has the potential to overcome this problem [22].

For isolation of protoplasts plant cell walls is subjected to enzyme digestion which wakens the cell walls, protoplasts are released and chromosomes are set free of cytoplasm. This step needs to be carefully optimized and depends on the nature of the plant material used, the relative concentration and the ratio

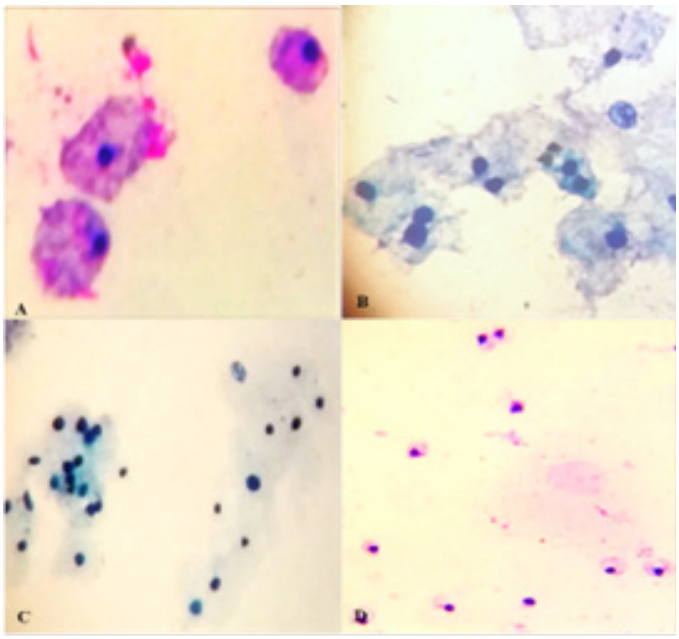

Fig. 1 Effect of different concentrations of cellulase: pectinase enzyme mixture on protoplast morphology. A Turgid protoplast formed at 5% cellulase: pectinase mixture after 3 hours incubation period B insufficiently digested cell wall observed at 1or 2% cellulase:pectinase mixture C sticky protoplast formed at 4% cellulase: pectinase mixture after 4 hours incubation period D Rupture or shrink protoplast formed at 4or 5% cellulase pectinase mixture after 4or 5 hours incubation period.

of digesting enzymes, and the duration of enzyme treatment.

In the present work mixture of cellulase (Advance enzyme Sun Magnetica, India) and pectinase (Himedia) was used in 1:1 proportion. As described in Table 3, the effect of the various concentrations (1, 2, 3, 4, 5 %) of cellulase and pectinase combination were studied to see their effect on the protoplast formation and morphology. For each concentration duration of enzyme treatment was also varied from 1 hour to 5 Hours. As seen in Table 3, the cellulase/pectinase mixture at very low concentrations (1 % and 2 %) does not release the protoplast in the mixture at any time duration due to inadequate digestion of cell wall (Fig. 1B). At 3 % cellulase/pectinase mixture, some protoplasts were released after 4 hours incubation period but the number of protoplasts formed was very less (5protoplasts) and when incubation period was increased to 5 hours, the number of protoplasts was decreased to 3. When the cells were treated with a mixture of 4% cellulase and pectinase, at 4 hours of incubation quite a good number of protoplasts (15) were formed. But they were forming aggregates or clusters (Fig. 1C) Similar types of aggerated

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Table 3. Effect of different cellulase / pectinase enzymes concentrations along with various time durations on protoplast formation

Cellulase: Pectinase (1:1) concentrations	Treatment durations (Hours)	Average number of protoplasts at 40X	Remark
1% (Cellulase : pectinase)	-	-	No protoplast formation at any treatment duration
2% (Cellulase : pectinase)	-	-	No protoplast formation at any treatment duration
3% (Cellulase : pectinase)	1	-	No protoplast formation
	2	-	No protoplast formation
	3	1	Protoplast formation is very less
	4	4.6	Protoplast formation is very less
	5	3	Protoplast formation is very less
4% (Cellulase : pectinase)	1	-	No protoplast formation
	2	-	No protoplast formation
	3	5.6	protoplasts stick together
	4	15.4	protoplasts stick together
	5	9.8	Protoplasts rupture
5% (Cellulase : pectinase)	1	-	No protoplast formation
	2	-	No protoplast formation
	3	23.8	Protoplast turgid and intact
	4	9.4	Protoplasts shriveled
	5	5.4	Protoplasts rupture

protoplasts were observed after 3 hours incubation period wherein, a number (6) of protoplasts were released in the mixture. After 5 hours of incubation at 4 % cellulase/pectinase mixture, leaf tissue released only 10 protoplast and most of them observed to be disintegrated. (Fig. 1D). Among all the cellulase/ pectinase concentrations highest number (24) of protoplasts released were reported at 5 % cellulase/ pectinase mixture after 3 hours incubation period and the protoplast formed were found to be turgid and intact (Fig. 1A). At 4 hours and 5 hours incubation period with 5 % cellulase/pectinase mixture the number of protoplasts formed were comparatively less i.e., 9 and 5respectively and most of them were shrivelled and ruptured suggesting a higher dose of treatment.(Fig. 1D).

Hence in all, low enzyme concentrations (1% and 2%) and shorter time duration (1 hour and 2 hours) of enzymatic incubation cause insufficient digestion of cell wall whereas, on the other hand, longer enzymatic treatment (4 hours and 5 hours) causes increased stickiness and disintegration of Protoplasts. Similar patterns with incubation period were seen during the separation of protoplasts from suspension cultures of wheat and poppies. A substantial percentage (25–30%) of metaphase protoplast isolation was seen after 3 hours of treatment with an enzyme solution (6% Cellulase (Onozuka R-10), 2% Rhozyme (Rhom and Haas), 2% Pectinase (Serva), and 5% Driselase).When the incubation period decreased below to 3 hours caused an inadequate digestion of the cell wall while, the longer enzymatic treatment caused increased stickiness and disintegration of chromosomes [10].

In *Betula* L. protoplast isolation was achieved by treating young leaf samples of the tree with 2.5% (v/v), Pectinase (Sigma P-9179) and 2.5% (w/v), Cellulase Onozuka R10 (Merck 102321) in buffer containing 75 mM KCl and 7.5 mM EDTA [4] for 3 to 5 h at room temperature. Protoplast isolation from shoot tips of the rose plant was reported to have taken place after the sample was incubated for 3 to 4 hours at room temperature in a solution of 5% cellulase, 1% pectolyase, and 0.01 M sodium citrate at pH 4.6 [19]. For comparative chromosomal analysis of 14 species of Lens culinaris Medik., root samples were treated with enzyme mixture cellulase Onozuka RS 1%, macerozyme R-10, 0.75%, pectolyase Y-23 0.15% and 1 mM EDTA) for 45–55 minutes at 37°C for protoplast preparation [12]. Therefor it was observed that the conditions for enzymatic digestion are plant specific.

Metaphase spread preparation

The quality of metaphase spread is decided by many factors such as, the distance from which the fixed mitotic cells are dropped onto the slide, the diameter of the opening of the pipette that dictates the size of the drops, the amount of evaporating fixative on the slide, and the temperature of the slide itself during evaporation of the fixative [7].

In the present work, we have tried four different (2', 3', 4', 5.2') heights to drop the protoplast suspension using a pasture pipette of 1mm diameter onto the ice-cold slide. At lower height such as 2', protoplast do not burst open to have metaphase spread whereas, when protoplast suspension was dropped from 3' and 4' height, the chromosomes get overlapped and squished together, which make staining and evaluation of metaphasic spread difficult. The best metaphase spread was obtained when protoplast suspension was dropped from 5.2' height. If the distance increased beyond 5.2', the chromosomes spread too far apart. And there was the possibility of merging the chromosomes of one cell with a neighboring cell. After dropping, slides were immediately transferred on to the hot plate adjusted at 87 ^{o}C for 15 min to evaporate the fixative and to have a quality metaphase spread. In the process of evaporation of fixative methanol evaporates first which causes shrinkage of the cell. Acetic acid being hygroscopic acquires water from the atmosphere and then water induces dramatic swelling of mitotic cells necessary for the production of well-spread metaphase [7]. Therefore, interaction in between acetic acid, water, and cellular proteins at the time of evaporation of fixative leads to chromosome stretching, the emergence of giemsa banding pattern, and metaphase spreading.

Giemsa staining

In the present investigation to stained metaphase spread, we have used Giemsa stain, as earlier study claims that chromosome arms, primary constrictions, and satellites are easily visible with Giemsa. Hence, completely dry metaphase spreads were stained with Giemsa for 10 minutes, which is found to be sufficient to stain the chromosomes more intensely with cleared morphological details.

Ideogram Formation

Chromosomes number was confirmed by analyzing 40 well-spread metaphases and images were captured using Olympus CX33 LED illuminated trinocular microscope(Fig. 2A). Software karyotype analysis V2.0 was used to take the measurements of the chromosomes and marked satellites present whenever on the chromosomes. Data recorded of long arm length, short arm length, total length, and ratio of long arm to the short arm was used by software to construct the ideogram. Finally, the software arranged all the chromosomes considering their centromere position and total chromosomal length (Fig. 2B). Analysis of the chromosomal spread in *G. indica*, assisted by the protoplast dropping method, in the current study confirmed 2n = 54, which is consistent with the chromosome number reported in a previous finding of *G. indica*. [4]. These 54 chromosomes include 18 pairs of metacentric, 8 pairs of sub-metacentric and 1 pair of sub-telocentric chromosomes. The ideogram represents four pairs of satellite chromosomes, satellites present on the long arm of three pairs of metacentric and one pair of submetacentric chromosomes.

CONCLUSION

The presented protocol is useful for obtaining uniformly distributed and morphologically intact chromosome spread which is a pre-requisite for molecular cytogenetics experiment. When compared to the squash spread approach, the protoplast dropping method creates a more uniform, clean spread and allows for complete tissue digestion before spreading. As a result, the methodology presented here provides a trustworthy way to prepare chromosomes and is appropriate for routine karyotype analysis of large sample quantities.

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