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Production and verification of lemon × acid lime hybrid populations *via* embryo rescue

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ABSTRACT

Interspecific hybridization between canker-tolerant Konkan Seedless lemon (*C. limon* Burm) and commercially grown Kagzi lime (*C. aurantifolia* Swingle) is significantly impeded by the occurrence of nucellar embryony. Hence, our work aimed to enhance hybrid seedlings' recovery and confirm their hybridity through polymorphic SSR markers. Results revealed quite a satisfactory germination (< 58%) from all the stages of embryos and media used, but younger and oldest embryos produced more than 87% germination on all three media tested. MS medium supplemented with 500 mg l-1 malt extract plus 1.5 mg l-1 GA3 and 20µl l-1NAA proved better for the survival of plantlets obtained from the culture of either aged or younger embryos. However, it was highest in plantlets obtained from the culture of 80-90 days old embryos. The seedlings obtained from culture 80-90 old embryos (DAP or days after pollination) on MS medium supplemented with 500 mg l-1 malt extract plus 1.5 mg l-1 GA3 and 20µl l-1 NAA had the highest root and shoot length at 30 days after culture (DAC). SSR loci CCSM4 and CAC-33 expressed the highest polymorphism, showing their ability to distinguish the hybrids of Konkan Seedless (lemon) × Kagzi lime (acid lime).

Keywords: *C. limon* Burm × *C. aurantifolia* Swingle, Nucellar, Polyembryonic, SSR.

INTRODUCTION

Lemon cultivar Konkan Seedless (*C. limon* Burm) is highly tolerant to citrus bacterial canker disease (CBCD), and bears seedless fruits round the year but its fruits have a thicker rinds and lower acidity and lower juice content than acid lime. On the other hand, acid lime (*C. aurantifolia* Swing) is one of the most important acid citrus fruits grown commercially in many countries including India, but its high susceptibility to CBCD makes this crop uneconomical, particularly in north and central parts of India. The hybrids of these two genotypes of the acid citrus group can have the practical possibility to produce canker-tolerant hybrids with good desirable traits. The presence of nucellar embryony is the main constraint to producing hybrids in these species. Apomictic seedlings are an annoyance in *Citrus* improvement, as zygotic embryos fail to stay alive due to powerful competition with apomictic embryos for food and space, and if they germinate, it is often tough to identify zygotic seedlings from the seedling population in absence of morphological markers.

To overcome this problem, zygotic embryo rescue at an early stage is required to eliminate nucellar seedlings. The response of citrus embryos to *in vitro* culture is influenced by the stage of embryo rescue, the media makeup, and the tested plant genotype (Yi*et al*., 20). Further, it was stated that the size of zygotic embryo and its survival have an inverse relationship with the number of embryos per seed (Soares Filho *et al*., 13). Production of nucellar embryos decreases the range of genetic variability, which further limits the extent of finding new genotypes through hybridization in polyembryonic *Citrus* species. Consequently, the determination of suitable embryo age for *in vitro* culture in particular cross combination is the most important task, as it assures the good germination of immature embryos, and their subsequent development as hybrid seedlings. Additionally, culture media also play a significant role in the *in vitro* germination of embryos, and subsequent plantlet growth and survival. Earlier, White medium (White, 19) was used as the basic medium for tissue culture, but subsequent modifications in its composition resulted in the development of MS medium (Murashige and Skoog, 10), Gamborg B5 medium (Gamborg *et al*., 5), MT medium (Murashige and Tucker, 11) and DKW medium (Driver and Kuniyuki, 4). In citrus, many researchers have tried to alter the media composition including pH, Agar, sucrose, malt, plant growth regulators concentrations (Carimi *et al*., 2), macro - and micro-nutrients, and vitamins of the MT medium (Moraes *et al*., 9) with fair to good success. Malt extract appears to play a vital role in embryo cultures of *Citrus* species (Molnar *et al*., 8). Besides, increasing the sucrose concentration and adding GA_3

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have been shown to improve the immature embryos germination rates in mandarin (Sykes and Lewis, 16). Thus, an appropriate developmental stage of zygotic embryo and culture media is required for successful embryo rescue, *in vitro* culture, and embryo germination for particular cross combinations.

Early identification of hybrids in citrus requires some heritable morphological traits like trifoliate leaves, anthocyanin and thorn but, such traits are limited, and cannot be used everywhere. In absence of suitable morphological markers, DNA molecular markers particularly the co-dominant markers such as the SSR markers are of great importance for the rapid assessment of hybrids (Sundaram *et al*., 15). So the present study aimed to optimize the embryo age and medial composition for high recovery of zygotic seedlings in the crosses of lemon and lime varieties with early establishing hybridity using SSR markers.

MATERIALS AND METHODS

Konkan Seedless (lemon) and Kagzi lime (acid lime) were used in this study as maternal and paternal parents, respectively. The fruit set was recorded 30 days after pollination (DAP). For embryo culture, developing hybrid immature fruits of Konkan Seedless × Kagzi lime were harvested at three embryo age i.e., 80-90, 110-120 and 130-140 days after pollination (DAP). The fruits were washed with running tap water, thereafter sterilized with sodium hypochlorite suspension for 10 min, and washed with sterile water followed by 0.1% teepol solution (15 min), and subsequently treated with a solution containing 0.1% each of bavistin and indofil along with 0.02% 8-HQC for 3 h. Fruits were then rinsed with distilled water thrice, and shifted to a laminar flow hood. Surface sterilization was done with $HgCl_2$ (0.1%) for 10 min followed by 3-4 washing with sterile double distilled water. After cutting the fruits, seeds were counted in every fruit before the embryo extraction. The zygotic embryos were removed from the micropylar end of the seed after removing the seed coat. Isolated zygotic embryos were cultured on MS medium (Murashige and Skoog, 11) supplemented with 500 mg l^{-1} malt extract (M1), MS supplemented with 500 mg l^{-1} malt extract plus +1.5 mg $^{-1}$ GA_3 and 20µl l^{-1} NAA (M2) and DKW medium (Driver and Kuniyuki, 4) supplemented with 1.5 mg l^{-1} GA_3 +2µM 2ip (M3). Additionally, 30 g sucrose l^{-1} was added to each medium. Before autoclaving at 121 ^{0}C for 21 min, the pH of the media was accustomed to 5.7. All cultures were incubated at 24 ± 2 ^{0}C with a 16 h photoperiod.

The date on which embryo germination appeared, was recorded for counting the days required for germination. Likewise, the date was also noted for achieving the highest germination of inoculated embryos. Growth data in terms of root length, plantlet height, and the number of leaves were measured on 10 randomly selected plantlets in each treatment 30 days after culture (DAC). Total DNA was extracted from the leaf of parents and progenies as suggested by Doyle and Doyle (3). Purification and quantification of DNA were done as per the method of Shareefa *et al*. (12). SSR loci were randomly selected from the National Centre for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/pmc/articles /PMC3810390) to test the polymorphism between the parents. Selected thirty-seven SSR primers were synthesized by Microgen. PCR amplification was done with 50 ng of genomic DNA, 0.5 µl *Taq* DNA polymerase, 5.0 µl of 1× PCR master mix (Bioscience), and 0.4 µl of each primer with a final volume of 10 µl in a 48-well PCR System C1000 Touch ™ Thermal cycler (Bio Rad, USA). The PCR cycling profile was 94 ^{0}C for 5 min, 35 cycles at 94^{0}C for 30 s, 56 ^{0}C for 30 s, 72^{0}C for 60 s, and a final extension at 72^{0}C for 10 min. An amount of 10µl amplified product was taken from the PCR tube containing amplified products, and loaded in the slots of the agarose gel, thereafter. Five micro litres of 100 bp ladder (Bioscience) were loaded in the first lane of each gel to decide size of the identified bands. Electrophoresis was carried out at 50 V for 2.5 h. The gels were photographed on a Gel Doc (C200, Azure Biosystem, USA).

Primers those generated polymorphic and reproducible banding patterns between parents for defined cross combinations were picked for final analysis. Thereafter, parents were analyzed along with progenies obtained from the culture of the embryo of different ages Plantlets having two bands or bands similar to male parents were regarded as hybrid ones. One embryo per test tube was cultured, and 32 to 48 embryos were cultured in each treatment. For growth observations, treatments were arranged in the factorial experiment in a Completely Randomised Design with five replications. Each replication consisted of 3 seedlings. Data were subjected to statistical analysis using SAS software version SAS 9.3(SAS Institute, CARY, NC, USA). *P* value ≤ 0.05 were considered significant.

RESULTS AND DISCUSSION

The number of seeds/fruit decreased with the increased age of fruits (Table 1), and earlier fruit harvesting (80-90 DAP) tended to produce the highest seeds/ fruit. The number of seeds with functional embryos also differed with the harvesting stage, and seeds obtained from immature fruits (130-140 DAP)

Table 1. Effect of embryo age on number of seeds per fruit and seed with and without functional embryos in Konkan Seedless (lemon) × ALC-2 (lime*)* crosses.

Embryo age (DAP)	Number of seeds/ fruit	Number of seeds obtained	Seed with functional embryo (%)	Seeds without embryo (%)
80-90	21.2	106	83.96	16.04
110-120	18.2	128	92.19	7.81
130-140	10.4	48	100.0	0.00

*DAP; Days after pollination

contained 100% functional embryos, while its lowest number (83.96% seeds) was found in fruits harvested earlier (80-90 DAP). A significant effect of the fruit harvesting stage on growth and number of embryos in citrus was also found by Spiegel-Roy and Kochba (14), and later it was supported by Carimi *et al*. (2) who additionally reported a single embryo in the central position at the micropylar apex at the more advanced developmental stage of the embryo sac. Further in the grapevine, more embryos were reported by Gray *et al*. (6), while embryos were rescued at a later stage.

The highest embryo germination was recorded, when younger embryos (80-90 days old) were cultured on the M3 medium, while it was lowest in 110-120 days old embryos cultured on the M2 medium (Table 2). The embryos of all ages, while cultured on either medium took 3 days to initiate germination except for the embryos cultured at 80-90 days old embryos on M3 medium which took 5 days. Likewise, germination was completed earliest, when the oldest embryos (130-140 DAP) were cultured either on the M1 medium or on the M3 medium. Younger embryos (80-90 DAP) and middle-aged embryos (110-120 DAP), while cultured on either M1 or M2 medium took the longest duration (11 days) to complete the embryo germination (Table 2). Overall, embryos rescued at 80-90 DAP, and cultured on the M3 medium produced the highest germinating embryos, but later plantlet survival was lower than culture on the other media (M1 and M2). Hitherto, variation in *in vitro* embryo germinations due to embryo age was reported in citrus (Turgutoglu *et al*., 18). Normally, embryo germination needs only the basal medium without plant growth regulators, because the mature embryo can synthesize the plant growth regulator itself. However, in our case, we used immature embryos, and supplementation of culture medium was compulsory due to the elimination of food storage tissue. DKW basal medium supplemented with 1.5 mg l^{-1} GA_3 + 2µM 2ip (M3) might be beneficial for the germination of all younger and older embryos, as these embryos might have a low amount of synthesized plant hormone, while the oldest embryos performed well in all media because they might have some synthesized food also. Embryo germination has been reported to be influenced by the composition of medium and embryo age in citrus fruits (Tan *et al*., 17; Turgutoglu *et al*., 18).

The highest survival of progenies in this cross was noticed, while grown from the culture of the

Table 2. Effect of embryo age and media on embryo germination, days took to initiate and complete embryo germination in Konkan Seedless (lemon) × ALC-2 (lime) crosses.

Treatment	Number of embryos cultured	Number of Embryo germinated	Days took to initiate germination	Days took to complete germination	Germination (%)	Survival (%)
			Embryo age (80-90 DAP)			
M1 medium	39	34	3	11	87.18	91.18
M2 medium	37	31	3	11	83.78	93.55
M3 medium	32	30	5	8	93.75	75.00
			Embryo age (110-120 DAP)			
M1 medium	42	29	3	11	69.05	79.31
M2 medium	48	28	3	11	58.33	75.00
M3 medium	38	30	3	9	78.95	70.00
			Embryo age (130-140DAP)			
M1 medium	16	14	3	4	87.50	57.14
M2 medium	16	14	3	6	87.50	78.57
M3 medium	16	14	3	5	87.50	64.29

*DAP - Days after pollination

youngest embryos on the M2 medium, which was followed closely by those on the M1 medium. The lowest plantlets survival at 30 days after inoculation was noticed in seedlings acquired from the culture of oldest embryos on M1 medium (Table 2).

Both embryo age and media affected the root length, plantlet height, and leaves/plantlets significantly at both the times namely 30th and 60th days of culture (Table 3). The combined effect of both embryo age and media showed significantly longest tap root in seedlings obtained from the youngest embryos, shifted into M2 medium at both 30th an 60th days after culture (DAC). The lowest root length was measured, while 110-120 days old embryos were cultured and seedlings were shifted M2 medium which did not differ significantly from the seedlings obtained from the culture of akin embryos on either M1 or M2 medium. Notwithstanding, an extreme increase in root length at 60th DAC compared to 30th DAC was found in plantlets obtained from culture of younger embryos and shifted in the M1 medium (Table 3). Both embryo age and media showed the longest plantlet at 30th days after inoculation in seedlings obtained from the culture of oldest embryos (130-140 DAP) shifted into M1 medium followed by those acquired from inoculation alike aged embryos and grown in M2 medium at 30th day of inoculation (Table 3). However, it was lowest in plantlets raised from the culture of youngest embryos (80-90 DAP) showing parity with those from akin aged embryos shifted in M3 medium and seedlings obtained from the culture of 110-120 days old embryos, grown in M2 medium at 30th DAC. Nevertheless, the higher increase at the 60th day over the 30th day of culture was found when plantlets acquired from the culture of 80-90 or 110-120 days old embryos, shifted in the M1 medium. The lowest enlargement at the 60th day as of the 30th day was noticed, while seedlings obtained from the culture of oldest embryos (130-140 days old) shifted in M3, having parity with all other treatments of oldest embryos (Table 3). The highest leaves per plant at 30 DAC were obtained in the progenies of 110-120 days old embryos grown in M3 medium though it was found statically similar to those obtained from the culture of oldest embryos (130-140 days old) grown in either medium. Overall, both M1 and M3 media exhibited their superiority for extension in shoot and root growth from 30 to 60 DAC in progenies acquired from 80-90 days old embryos, while more increase in root and shoot

Table 3. Effect of embryo age and media on the growth of plantlets obtained from Konkan Seedless (lemon) × ALC-2 (lime) crosses at 30 after culture (DAC).

Treatment	Root length (cm)	Shoot length (cm)	Leaves plant^{-1}	Increase in root length at 60 DAC over 30 DAC (%)	Increase in shoot length at 60 DAC over 30 DAC (%)	Increase in leaves at 60 DAC over 30 DAC (%)
Embryo age (80-90 DAP)						
M1	3.94cd	1.66e	2.2c	71.54a	156.19a	243.80b
M2	5.70a	3.26c	2.6bc	42.20de	102.05b	226.80bc
M3	3.52de	2.02de	2.2c	68.92ab	124.23ab	403.20a
Embryo age (110-120 DAP						
M1	3.32ef	1.80e	2.8abc	43.17cd	131.37ab	50.12de
M2	2.88f	2.04de	2.6bc	56.38bc	107.84b	133.60cd
M3	2.94f	2.36d	3.8a	37.59de	57.43c	-3.33e
Embryo age (130-140 DAP)						
M1	3.8de	3.44c	3abc	28.64ef	25.65cd	53.40de
M2	4.82b	3.98b	3.4ab	5.82g	22.16d	3.00e
M3	4.4cb	4.66a	2.8abc	16.42fg	7.25d	36.47e
LSD (P≤0.05)						
DAP	1.19	1.14	0.39	5.0	11.44	34.37
Media	1.19	1.14	0.39	5.0	11.44	34.37
DAP × Media	0.56	0.42	1.1	14.13	32.32	97.17

*DAC - Days after culture

*Each data represents the mean value of five samples. Values representing different letters are significant at P ≤ 0.05 (THST).

growth in progenies obtained from 110-120 days old embryos in M1 and M2 medium over 30 DAC. Progenies of 130-140 days-old embryos did better in the M1 medium in the case of root growth, while for shoot growth, all media worked well for extension of the shoot and root growth over 30 DAC.

For verifying the origin of progenies, in the studied population, 37 SSR loci were assessed for the polymorphism between Konkan Seedless lemon (female) and Kagzi lime (pollen donor) parents. Of these, two SSR loci (CCSM4 and CAC-33) realized polymorphism between these parents due to their distinct allelic patterns. The allele size 200 bp and 150 bp were found to be specific for parent P1 (female) and P2 (male) in the case of CCSM4, while, *vice versa* was found for CAC-33, respectively. To further check the validity of these primers, hybrids of all aged embryos were selected randomly screened along with their parents and found the presence of both the alleles in the hybrids, confirming their hybrid origin. It is also confirmed by these two polymorphic markers that all plantlets randomly selected from the bulk of plantlets obtained from the culture of different aged embryos, were of hybrid origin, and no nucellar seedlings were detected (Fig. 1A-C). Molecular screening is a necessity to unquestionably state that all the progenies are derived from hybrid embryos. Microsatellite or simple sequence repeats (SSR) markers have been usually considered dependable markers (Brown *et al*., 1), comfortable to use, exchangeable between laboratories, and not affected by changing environmental condition (Gupta *et al*., 7). Of these 37 SSR primers pairs, two primers pairs presented different banding patterns and were found highly polymorphic for lemon cv. Konkan seedless and acid lime cv. Kagzi lime. Both alleles were observed in the progenies of Konkan Seedless × Kagzi lime crosses, obtained from the culture of 80-90 DAP, 110-120 DAP, and 130-140 DAP using CCSM-4 and CAC-33 primers.

Based on embryo germination and growth of progenies, we can infer that DKW medium supplemented with 1.5 mg l^{-1} GA_3 + 2µM 2ip was better for germination in both younger (80-09 DAP) and older (110-120 DAP) embryos. The use of two SSR markers (CCSM-4 and CAC-33) was enough to identify the hybrid progenies of Konkan Seedless (lemon) × Kagzi lime (acid lime).

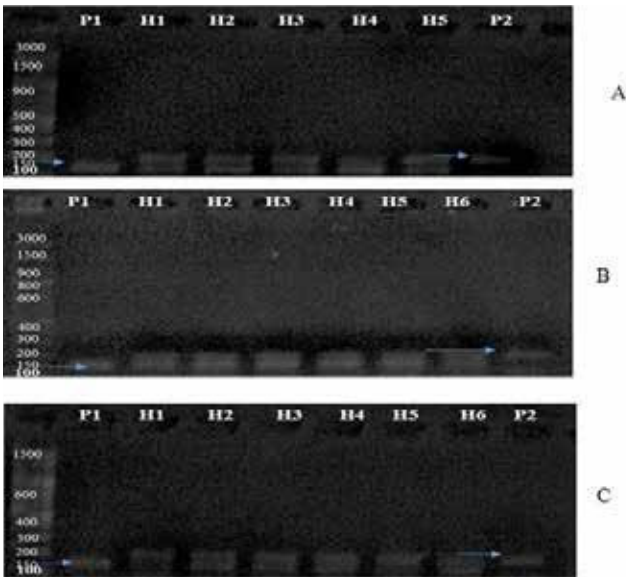

Fig. 1. Agarose gel profile of DNA amplification product obtained with SSR primers CAC33 *in vitro* raised plantlets obtained from 80-90 DAP (A), 110-120 DAP (B), and 130-140 DAP (C). Figure lanes are as follows: P1, female parent; H1-H6, Hybrids of Konkan Seedless (lemon) × Kagzi lime (acid lime); P2, male parent.

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AUTHORS' CONTRIBUTION

Identification of researchable areas supervision visualization (AKD); investigation, methodology (NS, AKD); formal analysis (RMS, AKD), writing original draft (AKD), review and editing (NS, RMS).

DECLARATION

The authors declare that they have no conflict of interest.

REFERENCES

1. Brown, S. M., Hopkins, M. S., Mitchell, S. E., Senior, M. L., Wang, T. Y., Duncan, R. R., Gonzalez-Candelas, F. and Kresovich, S.1996. Multiple methods for the identity of polymorphic simple sequence repeats (SSRs) in sorghum (*Sorghum* spp.). *Theor. Appl. Gen.* **93**: 190-98.

2. Carimi, F., De Pasquale, F. and Puglia, A. M. 1998.*In vitro*rescue of zygotic embryos of sour orange *Citrus aurantium* L. and their detection based on RFLP analysis. *Plant Breed.* **117:** 261-66.

3. Doyle, J. J. and Doyle, J.L. 1987. A Rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.***19:** 11-15.

4. Driver, J.A. and Kuniyuki, A. H. 1984. *In vitro*propagation of Paradox walnut rootstock. *HortSci*. **19** : 507-509.

5. Gamborg, O.L., Miller, R. A. and Ojima, K. 1968. Nutrient requirements of suspension culture of soybean root cells. *Exp. Cell Res.* **50**: 15-58.

6. Gray, D. J., Mortensen, J. A. and Benton, C. M. 1990. Ovule culture to obtain progeny from hybrid seedless bunch grapes. *J. Am. Soc. Hortic. Sci.* **115**: 1019-24.

7. Gupta, P. K., Balyan, H. S., Sharma, P. C. and Ramesh, B. 1996. Microsatellites in plants: a new class of molecular markers. *Curr. Sci.* **70**: 45-54.

8. Molnar, Z., Virág, E. and Ordog, V. 2011. Natural substances in tissue culture media of higher plants. *Acta Biol. Szeged.* **55**: 123-27.

9. Moraes, L. L., Vilegas, J. H.Y. and Lanc, F. M. 1997. Super critical fluid extraction of glycosylated flavonoids from passiflora leaves. *Phytochem. Anal.* **8:** 257-60.

10. Murashige, T. and Skoog, F. A. 1962. Revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15:** 473-97.

11. Murashige, T. and Tucker, P. H. 1969. Growth factor requirements of citrus tissue culture. *Proc. 1st Int. Citrus Symp*. **3**: 1155-161.

12. Shareefa, M., Singh, A. K., Manish, S. and Dubey, A. K. 2009. Differentiation of nucellar and zygotic seedlings in citrus using ISSR markers. *Indian J. Agric. Sci.***79**: 884-89.

13. Soares Filho, W.S., Vasquez Araujo, J. E., da Cunha, M. A. P., da Cunha Sobrinho, A.P. and Passos, O.S. 1992. Degree of polyembryony, size and survival of the zygotic embryo in citrus. Acireale Italy pp. 135-38.

14. Spiegel-Roy, P. and Kochba, J. 2005. Embryogenesis in citrus tissue cultures. *Adv. Biochem. Eng.* **16**: 27-48.

15. Sundaram, R. M., Naveenkumar, B., Biradar, S. K., Balachandran, S.M., Mishra, B., Ilyasahmed, M., Viraktamath, B. C., Ramesha, M. S. and Sarma, N. P. 2008. identification of informative ssr markers capable of distinguishing hybrid rice parental lines and their utilization in seed purity assessment. *Euphytica*, **163**: 215-24.

16. Sykes, S. and Lewis, W. J. 1996. Comparing Imperial Mandarin and Silverhill Satsuma mandarin as seed parents in a breeding program aimed at developing new seedless citrus cultivars for Australia. *Aust. J. Exp. Agric.* **36**: 731-38.

17. Tan, M., Song, J. and Deng, X. 2007. Production of two mandarin × trifoliate Orange hybrid populations *via*embryo rescue with verification by SSR analysis. *Euphytica* **157** :155-60.

18. Turgutoglu, E., Kurt, S. and Demir, G. 2015. Effect of GA_3 concentrations in basal medium on embryos germination of cleopatra mandarin × Carrizo citrange and cleopatra mandarin × Flying Dragon. *Ekin j. crop breed. genetic*. **1**:17-19.

19. White, P.R. 1943. *A Handbook of Plant Tissue Culture.* The Jacques Catlell Press, Lancaster, Pa. pg300

20. Yi, H. L., Deng, X. X. and Fu, C. H. 2000. Application of embryo rescue techniques in fruit crops. *J. Fruit Sci.***18**:224-28.

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