Control of Endogenous Bacterial Contamination and Micropropagation of a Traditional Table Banana (*Musa* spp. cv. Kanthali) of Bangladesh

香蕉组织培养过程中内生菌污染的控制

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摘 要 研究了体外培养一种孟加拉传统香蕉(*Musa* spp. Cv. Kanthali)的茎尖组织。茎尖的原始细胞表面经无菌处理(0.1% $HgCl_2$ 处理 12min),接种 6~15d 后外植体地下茎部分仍有微生物污染(大部分是细菌),杀死了 85% 的外植体。为确定无污染培养基,将等量外植体分别浸泡在含 400mg/L 氨苄青霉素和 200mg/L 庆大霉素(两种光谱抗生素)的培养基中 1h。结果表明,经抗生素处理的外植体完全没有污染,但培养 3 周后不能再生。进行二次继代培养后,其中一部分外植体吸收了培养基并胀大,颜色由苍白转变成浅绿或深绿。三次继代培养后数天,不再观察到外植体的生长,所有经抗生素处理过的外植体都开始死亡。在未经抗生素处理的活外植体中,单个茎发育的最佳培养基是:MS + 4.0mg/L BA + 0.5mg/L KT + 15% CW,平均生长时间为 18~21d,但再生率很低,只有 30%。茎细胞增殖的最佳培养基是:MS + 4.0mg/L BA + 2.0mg/L IAA + 15% CW,每个茎平均只萌发 3~4 个芽。最后,在添加 0.5mg/L IBA 的一半浓度的 MS 培养基中,体外培养茎最大生根率达到 90%。

关键词 香蕉, Kanthali, 体外, 茎尖, 抗生素
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Abstract Shoot tips of a traditional table banana (*Musa* spp. cv. Kanthali) of Bangladesh were evaluated for *in vitro* propagation. Initial surface sterilization (with 0.1% $HgCl_2$ for 12 minutes) of shoot tips was successful but microbial contamination (mostly bacteria) at the rhizomatous base of the explants was observed within 6~15 days after inoculation which eventually killed 85% of inoculated explants. So, for contamination free culture establishment explants were soaked in two broad spectrum antibiotics namely ampicillin and gentamicin. Cent percent contamination free cultures were established by soaking the explants in 400mg/L ampicillin or 200mg/L gentamicin for 1h. Antibiotic treated explants were found to be full contamination free but failed to regenerate after 3 weeks of culture. But some of them absorbed media for up to 2nd subculture and showed swelling of explants and some color changes from pale white to light/deep green. Finally, a few days after 3rd subculture, no growth of explants was observed and all treated explants eventually started to die. Among the untreated alive explants the best medium for single shoot development was MS + 4.0 mg/L BA + 0.5mg/L KT + 15% CW and average time required for shoot development was 18~21 days. But the regeneration percentage was very low (30%). The best medium for shoot multiplication was MS + 4.0 mg/L BA + 2.0mg/L IAA + 15% CW and only average 3~4 shoots were formed per shoot. Finally, *in vitro* proliferated shoots produced roots with maximum frequency (90%) in half strength of MS medium fortified with 0.5mg/L IBA.

Key words banana, Kanthali, *in vitro*, shoot tip, antibiotic

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Banana (*Musa* spp.) is the 4 th largest food crop in the world and affects lives of 400 million people[1]. Being parthenocarpic in nature, bananas and plantains are propagated vegetatively by means of small shoots or "suckers" from the parent plant. But the rate of multiplication through conventional method is slow and a number of viral diseases (bunchy top virus, banana streak virus) and other diseases are also transmitted to new generation. Thus, the productivity of fruits decreases and finally the yield become very poor which affects national economy of the country. In Bangladesh during the last few years the production of banana is decreasing gradually from 703314 tons in 1986 ~ 1987 to 628425 tons in 1997 ~ 1998[2]. It may be due to unavailability of healthy and virus free suckers. Normally four to five suckers are obtained per plants which are insufficient to replace the affected farms with healthy germplasm.

The development of micropropagation techniques has been a major focus of *Musa* research during the past two decades and such techniques have now been well established for large scale plant production[1,3,4]. Micropropagation of banana has been achieved using shoot tips[5] and from male floral apices[6]. Meristem culture for clonal propagation and virus eradication was done by Gupta in 1986[7]. Plant cells growing *in vitro* are considered to be under some degree of stress and may be predisposed to direct infection, even by bacteria not normally pathogenic to them[8]. The medium may contain many different bacterial nutrients, both organic constituents of the medium and exudates from the plant cells. Thus pathogens, endophytes, epiphytes and incidental contaminants may also occur and may interface with growth of the plant tissue[9]. Almost all plant pathogenic bacteria develop mostly in plants as parasites and partly in plant debris or in the soil as saprophytes. There are great differences among bacterial species, in the degree of their development in one or other environment. These bacteria enter plants through wounds made in roots and over winter in diseased plants or debris, vegetative propagative organs such as potato tubers and banana rhizome[10]. For *in vitro* propagation of banana, bacterial contamination is a great problem. Although initially surface sterilization works, later on microbial contamination at the base of the explant is observed within 7 to 15 days after inoculation. Bacterial growth is also observed around the explants in the culture media. Huge numbers of explants are destroyed in the culture due to endogenous bacteria[11].

Musa spp. cv. Kanthali (genome AAB) is a traditional table banana cultivar of Bangladesh[12]. The plant is only found in the southern part of the country and its population is continuously decreasing due to lack of commercial cultivation. Its production rate is relatively lower but the plant is more salt tolerant and disease resistant than other commercial cultivars. Therefore, the present study deals with the control of endogenous bacterial contamination and micropropagation of table banana cv. Kanthali from shoot tip explants. The aims of the experimental design addresses the following aspects of *in vitro* development:

- That sterilization procedure can be developed which will optimize tissue survival *in vitro*.
- That antibiotic treatment minimizes microbial contamination.
- That cytokinin and auxin ratios will facilitate tissue regeneration.

1 Materials and methods

1.1 Collection and preparation of explants

Banana cv. Kanthali (Genome AAB), a leading traditional cultivar of Bangladesh was used as the source material for culture establishment. The shoot tips along with a portion of rhizomatous tissue were used. In early of 2005, the experimental plants were collected from a village named Amtola in Police Station Batiaghata, Khulna and was very near from Khulna University campus. The explants were prepared by removing the outer layer of tissues from suckers with a clean knife. The pale white tissue blocks containing the shoot tip and rhizomatous bases were surface sterilized with 0.1% $HgCl_2$ for 12 minutes.

1.2 Antibiotic treatment

Microbial contamination (mostly bacteria) at the rhizomatous base of the explants was observed within 6 ~ 15 days after inoculation which eventually killed 85% inoculated explants. So, for contamination free culture establishment explants were soaked in two broad spectrum antibiotics namely ampicillin and gentamicin. Cent percent contamination free cultures were established by soaking the explants in 400mg/L ampicillin or 200mg/L gentamicin for 1 hour.

1.3 Growth responses

After the treatment with antibiotics the shoot tips of banana were inoculated in MS[13] medium with varying concentrations of hormonal supplements for single shoot formation. But all of them completely failed to regenerate

and some of them absorbed media for up to 2nd subculture. They showed swelling of explants and some color changes from pale white to light/deep green. Finally a few days after 3rd subculture, no growth of explants was observed and all treated explants eventually stared to die.

From the untreated alive explants, in all concentrations of BA tissue swelling and ball like structures were formed. But the control didn't respond at all. Highest percentage (40%) of single shoot was formed when explants were cultured on MS + 4.0mg/L BA + 0.5mg/L KT + 15% CW and the average length of shoot was 4.5 0.37cm. These single shoots were further subcultured on different concentrations of cytokinin (BA), auxin (IAA) and 15% CW for multiplication of regenerated shoots. The growth rate was very slow and 40 ~ 45 days after inoculation only highest 3 ~ 4 shoots were produced in MS medium containing 4.0mg/L BA + 2.0mg/L IAA + 15% CW. Finally, *in vitro* proliferated shoots produced roots with maximum frequency (90%) in half strength of MS medium fortified with 0.5mg/L IBA.

1.4 Computation and presentation of data

Data on different parameters from different treatments of shoot proliferation were recorded after 2 ~ 7 weeks of culture and those from different experiments of rooting were recorded after 1 ~ 3 weeks of culture.

The data presented in tables in the forms of percentage of explants showed proliferation/rooting for each experiment were computed by as follows:

$$\text{Percentage of explants showed proliferation} = \frac{\text{No. of explants formed shoots}}{\text{No. of explants cultured}} \times 100\%$$

$$\text{Percentage of explants formed roots} = \frac{\text{No. of explants formed roots}}{\text{No. of explants cultured}} \times 100\%$$

(Contaminated and dead explants were excluded)

In the table, the mean data of different replications of each treatment was accompanied by Standard error of mean as an only statistical measurement which was calculated as follows:

$$\text{Standard Error (S.E.)} = S/\sqrt{n}$$

Here,

S = Standard deviation

n = Number (viz. no. of explants, no. of culture)

$$\text{Standard deviation (S.)} = \frac{\sqrt{n\sum x^2 - (\sum x)^2}}{n(n-1)}$$

Where, n = Sample size

2 Results and discussion

The experiment was conducted at Plant Biotechnology Laboratory of Khulna University, Bangladesh during January to November 2005. One indigenous banana plant (*Musa* spp. cv. Kanthali) of Bangladesh was studied in order to establish suitable protocols for *in vitro* plant regeneration. For culture initiation, all the experimental explants were cultured on MS medium supplemented with different concentrations of cytokinins and auxins for promoting morphogenic responses. The results of the experiment are described as follows.

2.1 Sterilization procedure for explants

To overcome contamination problem surface sterilization of the explants was done with 0.1% (*W*/*V*) $HgCl_2$ for different durations to assess the contamination percentages and viability of the explants used for *in vitro* culture. Most of the cultured explants showed bacterial contamination within 3 ~ 10 days of inoculation if they were not sterilized. Maximum number of cultured explants showed bacterial infection singly and a few number of explants showed bacterial and fungal infection together. None of the shoot tip explants were found contamination free after 15 days when treated with 0.1% $HgCl_2$ for 1, 2, 4 and 6 minutes. Only 20% explants were found alive and contamination free without any tissue damage when treated for 12 minutes with the same concentration of $HgCl_2$. It was found that explants were killed with the increasing duration of treatment with 0.1% $HgCl_2$ solution. 5% ~ 25% explants were killed due to tissue injury when treated for 15 minutes but highest number of explants (40%) found contamination free(Fig. 1).

2.2 Effect of antibiotic for contamination free culture establishment

Surface sterilization result showed that only 20% shoot tip explants were found alived and contamination free when treated for 12 minutes. Most of the explants died within 6 ~ 15 days due to broad range of bacterial and fungal contamination. Hence, it was necessary to further sterilize the explants for 100% contamination free culture establishment. For that purpose explants were immersed in screened (with 0.22μm disposable filter) antibiotics (ampicillin and gentamicin) for different durations to ensure contamination free cultures. The results show that cent percent contamination free cultures could be obtained by soaking the explants in 400mg/L ampicillin or 200mg/L gentamicin for 1 hour (Table 1).

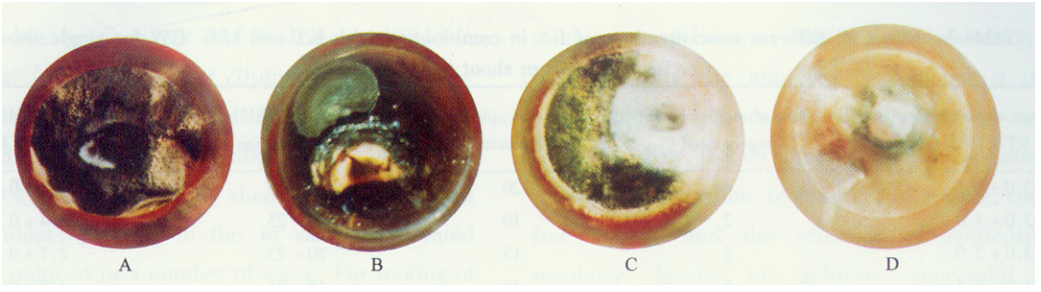

Fig. 1 Different mode of fungal and bacterial contamination on surface sterilized banana shoot tip explants after 3 weeks of culture
A & B：only fungal contamination；C：both fungal and bacterial contamination；D：only bacterial contamination.

Table 1 Effect of different concentrations of antibiotics immersion at different duration of time to ensure contamination free cultures

Antibiotics	No. of explants treated	Duration of treatment /min	Concentration of antibiotics/(mg/L)							
			50		100		200		400	
			Contamination free explants found							
			No	%	No	%	No	%	No	%
Ampicillin	10	30	1	10	3	30	4	40	7	70
	10	60	1	10	5	50	5	50	10	100
	10	100	1	10	5	50	6	60	10	100
	10	120	2	20	6	60	7	70	10	100
Gentamicin	10	30	1	10	6	60	6	60	10	100
	10	60	2	20	5	50	10	100	10	100
	10	100	2	20	6	60	10	100	10	100
	10	120	2	20	6	60	10	100	10	100

2.3 Growth responses of antibiotics treated explants

Antibiotic treated fully contamination free explants were cultured on MS medium supplemented with different kinds of growth regulators (auxin and cytokinin). But after 3 weeks of culture they were completely failed to regenerate. But some of them absorbed media for up to 2nd subculture and showed swelling of explants and some color changes from pale white to light/deep green. Finally a few days after 3rd subculture, no growth of explants was observed and all treated explants eventually stared to die (Table 2).

Table 2 Growth responses on antibiotics treated experimental shoot tip explants

Duration of treatment/min	Growth responses after 3 weeks of culture		Color change from primary explant after 3 week of culture		1 st subculture (3 weeks)	2 nd subculture (3 weeks)	3 rd subculture (3 weeks)
	Gentamicin/(mg/L) 200	Ampicillin/(mg/L) 400	Gentamicin/(mg/L) 200	Ampicillin/(mg/L) 400			
30	No shoot formation	No shoot formation	Pale white to deep green	Pale white to deep green	Enlargement of explants	Enlargement of explants	Explants died
60	No shoot formation	No shoot formation	Pale white to deep green	Pale white to light green	Enlargement of explants	Enlargement of explants	Explants died

2.4 Shoot initiation, multiplication and rooting of regenerated shoots from non-treated alived explants

Formerly, only 20% explants were found alive and contamination free after 2 weeks of culture on MS medium supplemented with different growth regulators. Those non-antibiotics treated alive explants showed quite promising growth responses on their growth medium (Fig. 2). The best medium for single shoot development was MS + 4.0mg/L BA + 0.5mg/L KT and average time required was 18 ~ 21 days. For the production of multiple shoots regenerated single shoots were cultured in MS media with different concentrations of auxin and cytokinin. The best medium for shoot multiplication was MS + 4.0mg/L BA + 2.0mg/L IAA + 15% CW. And with MS + 4.0 BA + 2.0 KT + 2.0 IAA + 15% CW some promising amount of rooting was also observed. But overall multiplication rate was also too low (40%) and only average 3 ~ 4 shoots were formed. Finally, *in vitro* proliferated shoots produced roots with maximum frequency (90%) in half strength of MS medium fortified with 0.5mg/L IBA (Table 3 ~ 5, Fig. 3).

Table 3 Effect of different concentrations of BA in combination with KT and 15% CW for single shoot formation from shoot tip explants

Growth regulators concentration/(mg/L) (BA + KT + 15% CW)	No. of single shoots regenerated	% of explant responded	Days required for shoot formation	Average length of shoots ($\bar{x}$ + S.E.)
3.0 + 0.5	4	20	20 ~ 25	2.0 ± 0.12
3.0 + 1.0	2	10	20 ~ 25	2.4 ± 0.12
3.0 + 2.0	3	15	20 ~ 23	2.7 ± 0.39
4.0 + 0.5	8	40	18 ~ 21	4.5 ± 0.37
4.0 + 1.0	6	30	15 ~ 21	4.2 ± 0.21
4.0 + 2.0	5	25	17 ~ 21	4.0 ± 0.17
5.0 + 0.5	3	15	16 ~ 21	3.0 ± 0.02
5.0 + 1.0	4	20	17 ~ 21	2.9 ± 0.02
5.0 + 2.0	2	10	16 ~ 21	3.1 ± 0.29

Table 4 Effect of different concentrations of BA in combination with KT, IAA and 15% CW for shoot multiplication

Hormonal supplements/(mg/L) (BA + KT + IAA + 15% CW)	No. of single shoots inoculated	No. of single shoot showed multiplication	% of explant responded	Average no. of multiple shoots/explant	Average time required/d	Intensity of rooting
3.0 + 0.5 + 2.0	10	1	20	2	45 ~ 50	−
3.0 + 1.0 + 2.0	10	1	10	2	45 ~ 50	−
3.0 + 2.0 + 2.0	10	2	20	2	45 ~ 50	+
4.0 + 0.5 + 2.0	10	1	10	3	40 ~ 45	−
4.0 + 1.0 + 2.0	10	2	20	2	40 ~ 45	−
4.0 + 2.0 + 2.0	10	3	30	2	40 ~ 45	+ +
5.0 + 0.5 + 2.0	10	1	10	2	40 ~ 45	−
5.0 + 1.0 + 2.0	10	2	20	2	40 ~ 45	−
5.0 + 2.0 + 2.0	10	1	10	2	40 ~ 45	+

" + "indicates very few amount of rooting;" + + "indicates sufficient amount of rooting;" − "no root formation

Table 5 Effect of different concentrations of IBA in half strength MS medium on adventitious root formation of the *in vitro* grown shootlets

Hormonal supplement/(mg/L) IBA	% of shootlets rooted	No. of roots/shootlet ($\bar{x}$ ± S.E.)	Average length of roots/cm ($\bar{x}$ ± S.E.)	Days to emergence of roots
0.0 (Control)	40	2.1 ± 0.35	2.9 ± 0.14	15 ~ 20
0.1	60	2.9 ± 0.16	3.4 ± 0.22	12 ~ 15
0.2	80	4.0 ± 0.12	4.2 ± 0.10	12 ~ 15
0.5	90	4.3 ± 0.23	4.7 ± 0.26	10 ~ 15
1.0	80	3.2 ± 0.14	4.0 ± 0.37	15 ~ 20

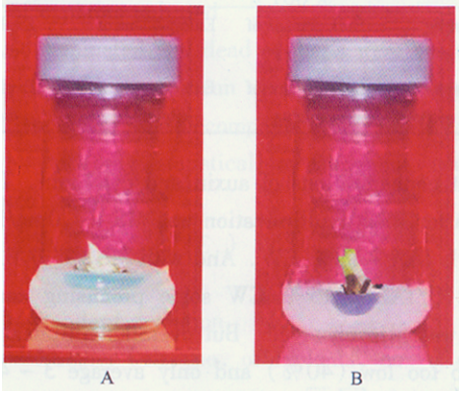

Fig.2 Establishment of 100% contamination free cultures on antibiotic treated shoot tip explants (A) and no shoot induction on MS medium supplemented with 4.0mg/L BA + 0.5mg/L KT + 15% CW (B) after 3 weeks of culture.

The experimental results indicated that initial surface sterilization of shoot tips was successful but microbial contamination at the rhizomatous base of the explants was observed within 6 ~ 15 days after inoculation which eventually killed 85% inoculated explants. The situation was very similar with Hadiuzzaman *et al* (2001)[11]. Identification of endophytic bacteria and their elimination from banana tissue culture was also done by some other researchers[14-17]. From the untreated alived explants different types of cytokinin, auxin and their concentrations significantly influenced shoot multiplication and elongation. Furthermore, the joint effect of BA and IAA increased shoot elongation compared to BA alone. The optimum BA concentration does not vary significant among researchers. For example, BA at 22.2μmol/L was

optimal in studies by Cronauer and Krikorian (1984)[5] and Jarret *et al* (1985)[18] and at 20μmol/L in a study by Vuyslteke (1989)[1]. Wong (1986)[19] stated that 44.4μmol/L BA reduced shoot multiplication. Arinaitwe *et al* (2000)[20] mentioned that shoot proliferation was cultivar dependent. Rooting in the *in vitro* proliferated shoots can be induced in a number of ways. For rooting of banana and plantain, Novak *et al* (1990)[21], Banerjee and De-Langhe (1985)[3] used half strength MS + 1.0μmol/L IBA and Hossain (1997)[22] used half strength MS + 2.0mg/L IBA. On the other hand, Cronauer and Krikorian (1984)[5] used hormone-free MS medium for rooting of banana shoots. They also used IAA, IBA or NAA in different concentrations for root induction and reported that addition of activated charcoal (0.25 ~ 2.5mg/L) increased the average root length but not root number. Banerjee *et al* (1986)[23] found that regenerated shoots formed roots in MS solid medium with half strength of macro-salts with 0.2mg/L IBA in banana and plantain. In the present investigation, auxin free media and media containing one type of auxin (IBA) at different concentrations was used to regenerate roots under *in vitro* condition.

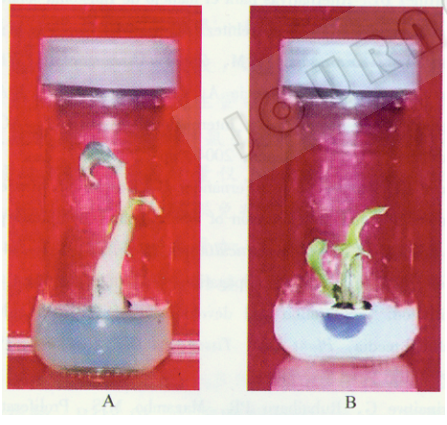

Fig.3 Single shoot induction on MS medium supplemented with 4.0mg/L BA + 0.5mg/L KT + 15% CW (A) and multiplication of shoots on MS medium supplemented with 4.0mg/L BA + 2mg/L IAA + 15% CW (B)

3 Conclusion

This study examined the application of micropropagation protocols to assist germplasm conservation of a traditional cultivar table banana [*Musa* spp. cv. Kanthali (Genome, AAB)] of Bangladesh. This has implications for commercial explant production in large scale as it generally produces 5 ~ 6 suckers from a mature mother plant per year. And for *in vitro* propagation of banana, bacterial contamination is a great problem. So, the aim was to establish a protocol for contamination free culture development using shoot tip explants. Key factors investigated in this study include different sterilization techniques to ensure contamination free culture and the selection of appropriate growth regulator levels to achieve successful *in vitro* regeneration.

Shoot tips of banana (*Musa* spp. cv. Kanthali) were evaluated for *in vitro* propagation. The initial surface sterilization (with 0.1% $HgCl_2$ for 12 minutes) was successful but microbial contamination at the base of the explant was observed within 6 ~ 15 days after inoculation which eventually killed 85% inoculated explants. So, for contamination free culture establishment explants were soaked in 200mg/L gentamicin or 400mg/L ampicillin for 1 hour. Antibiotic treated explants found to be full contamination free but failed to regenerate after 3 weeks of culture. But some of them absorbed media for up to 2nd subculture and showed swelling of explants and some color changes from pale white to light/deep green. Finally a few days after 3rd subculture, no growth of explants was observed and all treated explants eventually stared to die. Among the untreated alived explants the best medium for single shoot development was MS + 4.0mg/L BA + 0.5mg/L KT + 15% CW and average time required for shoot development was 18 ~ 21 days. But the regeneration percentage was very low. The best medium for shoot multiplication was MS + 4.0mg/L BA + 2.0mg/L IAA + 15% CW and average time required for production of multiple shoots from single shoot was 40 ~ 45 days. Multiplication rate was also too low and only average 3 ~ 4 shoots were formed in that particular concentration. The *in vitro* proliferated shoots produced roots with maximum frequency in half strength of MS medium fortified with 0.5mg/L IBA.

The interest in *Musa* spp. cv. Kanthali both for domestic and export sales would be increased in future. It is essential that an appropriate method to expedite introduction to cultivation be determined. The success of *in vitro* propagation methods reported that the experimental plant can be tissue cultured with further plant growth regulators experimentation. Successful micropropagation will be of major benefit to the Agricultural Biotechnology making conventional breeding method unnecessary thus greatly assisting in the

conservation of this unique Bangladeshi plant.

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LIST OF ABBREVIATIONS

BA 6-Benzyl adenine
CW Coconut water
IAA Indole-3-acetic acid
IBA Indole-3-butyric acid
KT Kinetin (6-furfuryl amino purine)
MS Murashige & Skoog (1962) medium
NAA α-Napthaleneacetic acid
W/V Weight per volume

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