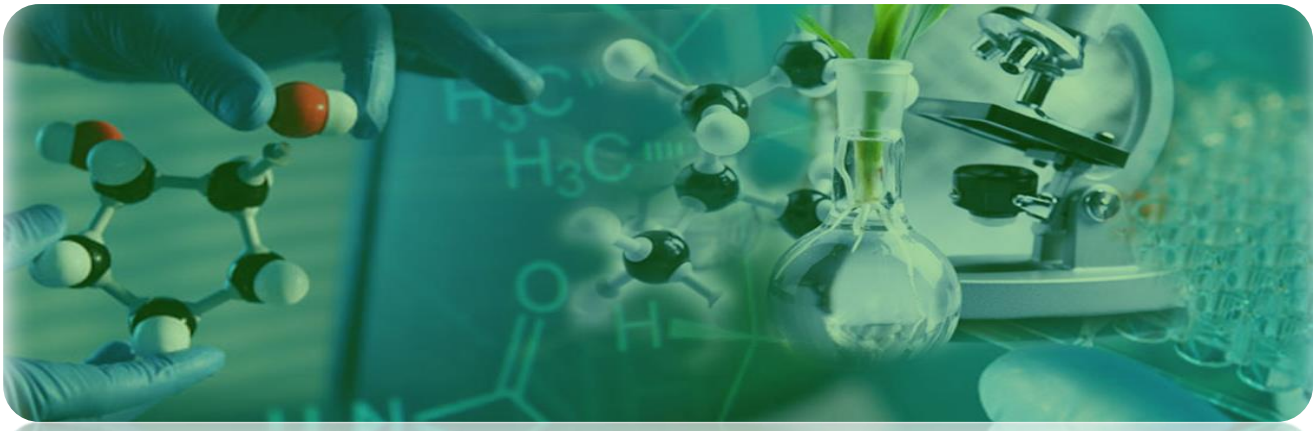
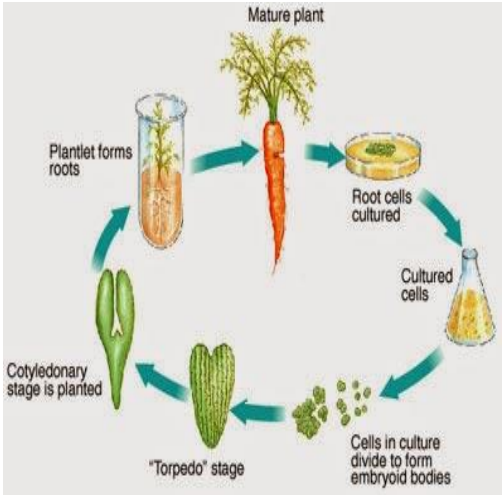
Elementary Plant Biochemistry & Biotechnology





2. Elementary Plant Biochemistry and Biotechnology (HBB 100) 3(2+1)

Carbohydrates: Occurrence classification and structure, physical and chemical properties of carbohydrates, isomerism, optical activity, reducing property, reaction with acids and alkalis, ozone formation. Lipids: Classification, important fatty acids and triglycerides, essential fatty acids. Physical and chemical control of oils, their rancidity, phospholipids, types and importance. Plant pigments – structure and function of chlorophyll and carotenoids, sterols, basic structure, role of brassinosteroles in plants. Proteins: Classification, function and solubility, amino acids – classification and structure, essential amino acids, properties of amino acids, colour reactions, amphoteric nature and isomerism; structure of proteins primary, secondary tertiary and quaternary properties and reaction of proteins. Enzymes: Classification and mechanism of action; factors affecting enzyme action, co-factors and coenzymes. Vitamins and minerals as co-enzymes/co-factors. Carbohydrate metabolism –glycolysis and TCA-cycle; metabolism of lipids, fatty acid oxidation, biosynthesis of fatty acids, electron transport chain, bioenergetics of glucose and fatty acids, structure and function of nucleic acid replication, transcription and translation. History of biotechnology. Fundamental principles, micro-propagation and scope for commercialization. Application of micro-grafting in horticultural crops, meristem culture, anther culture, pollen culture, embryo culture, callus culture, cell culture, somoclonal variation, protoplast isolation, culture, fusion and applications. Cryopreservation. Genetic engineering. Future scope and present trends. Importance of biotechnology in horticulture.

***Practical**: Preparation of standard solutions and reagents. Carbohydrates – qualitative reaction, estimation of starch, reducing and non-reducing sugars; reaction of proteins, estimation of proteins by Lowery method. Estimation of free fatty acids; determination of iodine number of vegetable oils. Vitamins – estimation of ascorbic acid. Paper and thin layer chromatography. Sterilization techniques – composition and preparation of media micropropagation of tomato. Callus culture, sub-culturing, induction of rooting-techniques in hardening.*

Lecture.20

Lipases and Phospholipases, β-Oxidation of fatty acids and energetics of β -Oxidation

Lipids constitute one of the four major classes of compounds that are found in living systems. The lipids of metabolic significance include triacylglycerol, phospholipids and the products of lipid metabolism such as free fatty acids and glycerol.

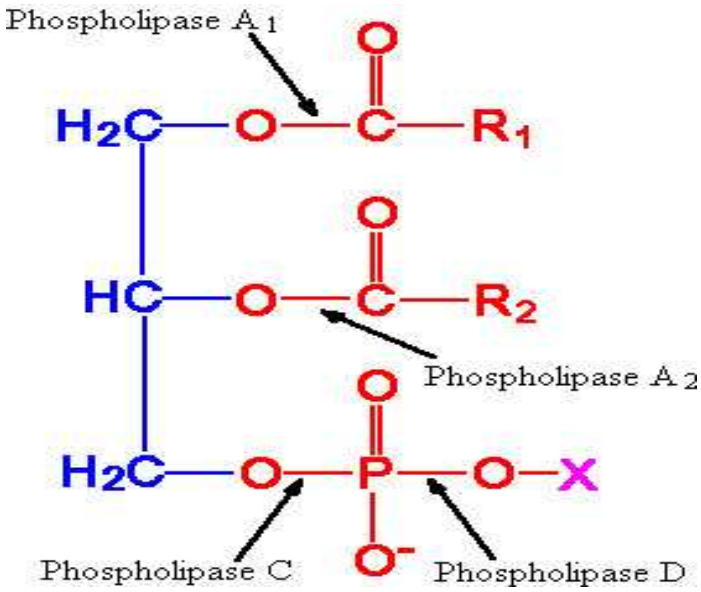
Lipases

- Triacylglycerols or triglycerides undergo hydrolysis by **lipases** to form glycerol and fatty acids, which undergo further oxidation generating energy.
- Lipases have been reported to be present in **dry seeds of some species, e.g. castor bean, Scots pine and Douglas fir** but at a low level, or absent in others e.g. apple.
- In most cases of seeds, **following imbibitions**, there appears to be a **rise in lipase activity** but whether this increase is due to the *de novo* synthesis of the enzyme or activation of existing lipases has not been determined.
- A **decline in lipase activity** is always associated with decline in acylglycerol reserves.
- In castor bean, as in many other fat-storing seeds, free fatty acids do not accumulate, but are rapidly degraded and converted to carbohydrate within the endosperm.
- In other seeds such as **germinating seeds of oil palm** (*Elaeis guineensis*), a different pattern of fat mobilization can be observed.
- The products of lipid catabolism are transported via specialized structures called **haustorium** through its vascular system.

- Lipases are generally **non-specific** and can hydrolyse a wide variety of triacylglycerols
- They initiate digestion by hydrolyzing triacylglycerols to form free fatty acids and 1, 2-diacylglycerols.
- Complete hydrolysis of triacylglycerols produces **glycerol and fatty acids.**
- Lipase hydrolyses easily the terminal fatty acids to produce 2-monoacyl glycerol as major

Phospholipases

- Phospholipases are the hydrolytic enzymes acting on phospholipids and splitting into different products.
- There are four types of phospholipases known as **phospholipase A_1, phospholipase A_2 or B_1, phospholipase C and phospholipase D.**



Phospholipase A

- Phospholipase A is present in large amounts in **snake venom and human pancreas.**

- It is also designated as **phospholipase A_1.**
- It catalyses the hydrolysis of the fatty acids in the 2 or β-position of the phospholipids.
- Though this enzyme attacks on glycerophosphatides, it is fairly specific for phosphatidyl choline (lecithin).
- The enzyme is relatively stable to heat (below pH 7.0).
- The product of the hydrolysis, a **lysolecithin**, (monoacylphosphoryl choline) has a powerful **hemolytic activity**.

Phospholipase B (A_2)

- It is otherwise termed as **lysophospholipase** and widely distributed in nature often in association with phospholipase A.
- Phospholipase B is also designated as **phospholipase A_2** since it acts on the lysolecithin (the product obtained from phospholipid by the action of phospholipase A_1).
- The action of this enzyme following that of phospholipase A yields **glycerophosphorylcholine** as the final product.

Phospholipase C

- Phospholipase C is mostly found in the plant kingdom but it may also be present in some animal tissues and venoms.
- It catalyses the liberation of a 1,2-diacylglycerol and phosphorylcholine from phosphatidylcholine.
- Phosphorylcholine is also liberated from sphingomyelin by this enzyme.

Phospholipase D

- Phospholipase D, an enzyme described mainly in plants catalyses the **hydrolysis of choline** from phosphatidylcholine leaving phosphatidic acid.

Oxidation of fatty acids

Fatty acids obtained by hydrolysis of fats undergo different oxidative pathways designated as alpha (α), beta (β) and omega (ω) pathways.

α-oxidation

- α-Oxidation of fatty acids has been found in certain tissues especially in **brain tissue of mammals and plant systems.**
- It does not require CoA intermediates and no high-energy phosphates are generated.
- This type of oxidation results in the **removal of one carbon** at a time from the **carboxyl end of the fatty acid**.
- The physiological role of α-oxidation in plants is not yet fully established but it has been suggested that it may be **involved in the degradation of long chain fatty acids** as observed in many animal tissues.
- α-Oxidation is clearly the **main source of the odd-carbon fatty acids** and their derivatives that occur in some plant lipids.
- In this process, sequential removal of one carbon at a time from free fatty acids of chain length ranging from C_{13} to C_{18} occur.

ω-Oxidation

- ω-Oxidation is normally a very minor pathway brought about by **hydroxylase enzymes** involving **cytochrome P-450** in the **endoplasmic reticulum.**

- Fatty acids with oxygen function (alcoholic or carboxyl) at the methyl terminal end (ω-end) are formed by ω-oxidation and frequently occur as constituents of **cutin and suberin.**

- The requirements for the oxygenase-mediated conversion of a ω-methyl fatty acyl CoA into a ω-hydroxymethyl fatty acyl CoA are **molecular oxygen,** reduced pyridine nucleotide and **a non-heme iron protein** in higher plants.

β-Oxidation of fatty acids

In 1904, Franz Knoop made a critical contribution to the elucidation of the mechanism of fatty acid oxidation and demonstrated that most of the fatty acids are degraded by oxidation at the **β-carbon.**

- β-Oxidation of fatty acids takes place in **mitochondria.**

- Fatty acids are activated before they enter into mitochondria for oxidation.

Activation of fatty acids

- Fatty acids are converted into active intermediate in a reaction with **ATP and coenzyme A.**

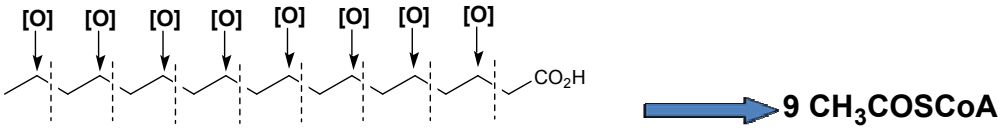
- A **thioester linkage** between the carboxyl group of a fatty acid and the sulfhydryl group of coenzyme A is formed with the hydrolysis of ATP.

- This activation reaction takes place on the **outer mitochondrial membrane** catalysed by **acyl CoA synthetase**.

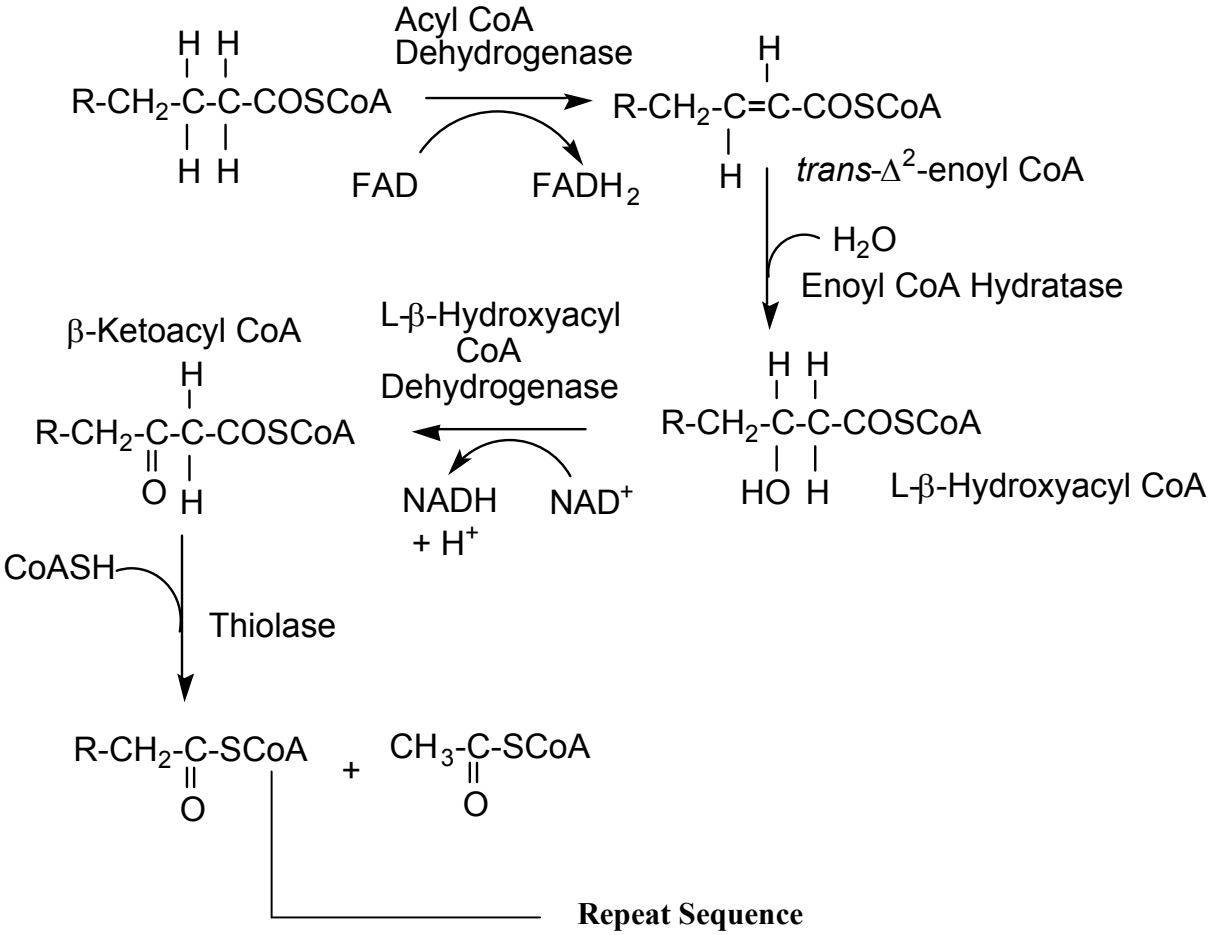
- Several **acyl CoA synthetases** each specific for fatty acids of different chain length are present in the membrane of mitochondria.

Beta Oxidation

- **Cleavage of fatty acids to acetate in tissues**
- **Occurs in mitochondria**

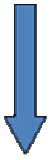


Beta Oxidation Reaction Sequence



Complete Beta Oxidation of Palmitoyl CoA

CH_3CH_2--CH_2CH_2--CH_2CH_2--CH_2CH_2--CH_2CH_2--CH_2CH_2--CH_2CH_2--$CH_2COSCoA$



7 Cycles

$$8\ CH_3COSCoA + 7\ FADH_2 + 7\ NADH + 7\ H^+$$

Penetration of long chain fatty acids into mitochondria

- Long chain acyl-CoA molecules do not readily get into the inner mitochondrial membrane and are carried across the inner membrane by conjugating with **carnitine** (β-hydroxy γ-trimethyl ammonium butyrate), a zwitterionic compound formed from lysine.
- Activation of lower fatty acids and their oxidation within the mitochondria occur independently of carnitine, but long-chain acyl CoA will become oxidised unless they form acylcarnitines.
- The acyl CoA combines with carnitine in the presence of **carnitine acyltransferase I,** which is bound to the outer mitochondrial membrane.
- **Acylcarnitine** is transported in, coupled with the transport out of one molecule of carnitine.
- The acylcarnitine then reacts with coenzyme A catalyzed by **carnitine palmitoyl transferase II,** located on the inside of the inner membrane.
- Acyl CoA is reformed in the mitochondrial matrix and carnitine is liberated.

Oxidation

A saturated acyl CoA is oxidised by a **recurring sequence of four reactions**

- Oxidation in presence of FAD, hydration, oxidation in presence of NAD^+, and thiolysis by CoASH.
- In β-oxidation, **2 carbons are cleaved** at a time from acyl CoA molecules, starting from the carboxyl end.
- The chain is **broken** between the **α-and β**-carbon atoms.
- The two-carbon units formed are acetyl CoA.

i) The first reaction in β-oxidation of acyl CoA is the formation of *trans* Δ^2-enoyl CoA or α, β-unsaturated acyl CoA in presence of acyl-CoA dehydrogenase and the coenzyme, FAD.

ii) The next step is the **hydration of the double bond** between C-2 and C-3 by enoyl CoA hydratase with the formation of β-hydroxy acyl CoA.

iii) In the third step, the β-hydroxy acyl CoA is **dehydrogenated** in the presence of **β-hydroxy acyl CoA dehydrogenase** and NAD^+ forming β -ketoacyl CoA.

iv) In the last step of β-oxidation, β-ketoacyl CoA reacts with coenzyme A in the presence of the enzyme, **thiolase.**

The products of this reaction are acetyl CoA and an acyl CoA containing **two carbons less than the original acyl CoA molecule** that underwent oxidation. By the above steps of β-oxidation fatty acids are completely degraded to acetyl CoA units. The acetyl CoA formed from fatty acids can be oxidised to carbon dioxide and water via citric acid cycle.

Energetics of β oxidation

The energetics or the energy conserved in terms of ATP by oxidation of a molecule of palmitic acid is given below:

- Palmitic acid (16 carbons) undergoes β-oxidation forming eight molecules of acetyl CoA by undergoing **seven β-oxidation spirals.**
- When one cycle of β-oxidation takes place, one molecule of $FADH_2$, one molecule of NADH and one molecule of acetyl CoA are produced.
- Electrons from these reducing equivalents ($FADH_2$ and NADH) are transported through the **respiratory chain in mitochondria** with simultaneous regeneration of high-energy phosphate bonds.
- Mitochondrial oxidation of $FADH_2$ eventually results in the net formation of about 1.5 ATP.
- Likewise, oxidation of electrons from NADH yields 2.5 molecules of ATP. Hence, a total of **four ATP molecules** are formed per cycle and **ten molecules of ATP** are formed through Krebs's cycle from each molecule of acetyl CoA.

8 Acetyl CoA through TCA cycle yield (8x10)	= 80 ATP
7 β-oxidation spiral reactions yield (7x4)	= 28 ATP

Total	**108 ATP**

ATP utilized in the initial step	= 2 ATP

Hence, **complete oxidation of palmitic acid** yields **106 ATP.**

Oxidation of monounsaturated fatty acids

- Oxidation of monounsaturated fatty acids follows many of the reactions of saturated fatty acids except the requirement of **two additional enzymes, an isomerase and a novel reductase.**

- Reactions of monounsaturated fatty acid are explained by considering the oxidation of a C-16 unsaturated fatty acid, palmitoleic acid, having a single double bond between C-9 and C-10 .

- Palmitoleic acid is activated and transported across the inner mitochondrial membrane in the same way as saturated fatty acids.

- Palmitoleoyl CoA undergoes three cycles of degradation as in β oxidation. But the *cis* Δ^3 decenoyl CoA formed after the third cycle does not serve as a substrate for acyl CoA dehydrogenase.

- The presence of a double bond between C-3 and C-4 prevents the formation of another double bond between C-2 and C-3.

- **An isomerase** converts the *cis* double bond into a *trans* double bond and shifts the position of double bond between C-2 and C-3.

- The subsequent or follow up reactions are those of the β oxidation pathway in which the *trans* Δ^2 decenoyl CoA is a regular substrate.

Oxidation of polyunsaturated fatty acids

The oxidation of a polyunsaturated fatty acid, linoleic acid, with *cis*-Δ^9 and *cis*-Δ^{12} double bonds is considered.

- The *cis*-Δ^3 double bond formed after three rounds of β-oxidation is converted into a trans double bond by the **isomerase.**
- This permits one more round of β-oxidation.

- The acyl CoA produced by four rounds of β-oxidation of linoleic acid contains a *cis*-Δ^4 double bond, which undergoes dehydrogenation by **acyl CoA dehydrogenase** yielding *trans* Δ^2, *cis*-Δ^4 dienoyl intermediate.
- This intermediate is not a substrate for the next enzyme in the β-oxidation pathway.
- This intermediate is converted into a ***trans* Δ^3 enoyl CoA** to the trans Δ^2 form, an intermediate generally found in β-oxidation pathway and results in complete oxidation of the fatty acid

Questions

Choose the correct answer

1. Triglycerides undergo hydrolysis by

a. Protease b. fatty acylase c. Thiolase d. Lipases

Ans: Lipases

2. Phospholipase A is present in large amounts in

a Plants. b. Animal tissues c. Snake venom d. Insect

Ans: Snake venom

3. ---------- catalyzes the hydrolysis of choline

a. Phospholipase A b. Phospholipase B c. Phospholipase C d.Phospholipase D

Ans: Phospholipase D

4. Complete oxidation of palmeticacid yields ----------

a. 108 b. 106 c. 100 d.104

Ans: 106

State True or False

5. The products of lipid catabolism are transported via specialized structures called haustorium.

Ans: True

6. There is an increase in lipase activity following imbibitions in most cases of seeds.

Ans: True

7. Phospholipase B (Az) is otherwise termed as lyso phospbolipase.

Ans: True

Write short notes

8.α-oxidation

9. Phospholipids A

10. Activation of fatty acids

Lecture.21

Fatty acid and triacyl glycerol biosynthesis

Biosynthesis of fatty acids

- It was thought that fatty acid biosynthesis occurred by **reversal of the β -oxidation pathway.**
- On the contrary, it occurs by a separate pathway that differs from β-oxidation in several ways.

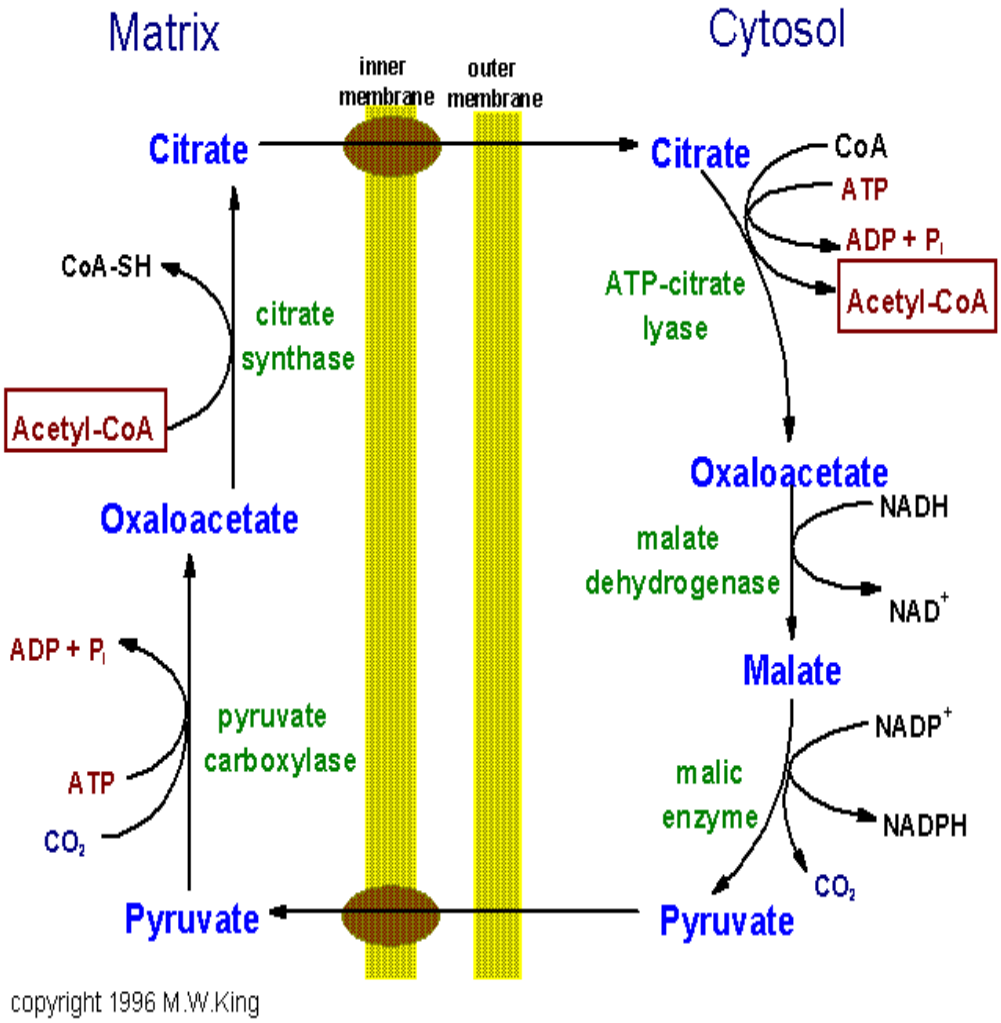
i. Synthesis takes place in the **cytosol,** in contrast with degradation or oxidation, which occurs in the **mitochondrial matrix**.

ii. Intermediates in fatty acid synthesis are covalently linked to the sulfhydryl group of an **acyl carrier protein (ACP)** whereas intermediates in fatty acid breakdown are bonded to coenzyme A.

iii. The enzymes of fatty acid synthesis in animals are **joined in a single polypeptide chain called fatty acid synthase.** In contrast, the degradative enzymes do not seem to be associated. Plants employ separate enzymes to carry out the biosynthetic reactions.

iv. The reductant in fatty acid synthesis is **NADPH,** whereas the oxidants in fatty acid oxidation are NAD^+ and FAD.

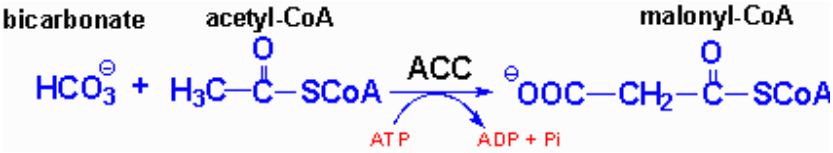
Pathway for the movement of acetyl-CoA units from within the mitochondrion to the cytoplasm for use in lipid and cholesterol biosynthesis.



The following seven steps are involved in fatty acid biosynthesis.

Formation of malonyl CoA

The synthesis of malonyl CoA from acetyl CoA is catalyzed by acetyl CoA carboxylase having biotin as prosthetic group. The production of malonyl CoA is the initial and controlling step in fatty acid synthesis. In this reaction, bicarbonate serves as a source of CO_2. The reaction takes place in two steps, namely carboxylation of biotin involving ATP and transfer of the carboxyl group to acetyl CoA resulting in malonyl CoA.



- **Acetyl CoA carboxylase** plays a key role in regulating fatty acid metabolism and the same is inactivated by phosphorylation.

ii) Formation acetyl and malonyl ACP

Acetyl transacylase and malonyl transacylase catalyze the formation of acetyl ACP and malonyl ACP respectively. Acetyl transacylase can transfer acetyl as well acyl groups whereas malonyl transacylase is highly specific.

Acetyl transacylase

Acetyl CoA + ACP → acetyl - ACP + COASH

Malonyl transacylase

Malonyl CoA + ACP → Malonyl - ACP + COASH

iii) Formation of acetoacetyl - ACP (β-ketoacyl ACP)

- Acetyl ACP condenses with malonyl ACP to form acetoacetyl ACP.
- Carbondioxide is eliminated from malonyl ACP.

iv) Reduction of β-ketoacyl ACP to β-hydroxyl acyl ACP.

- The β- keto group in acetoacetyl ACP is reduced by **NADPH-dependent β-ketoacyl reductase.**

v) Formation of unsaturated acyl ACP.

The β-hydroxyl group combines with the hydrogen atom attached to the γ -carbon and a water molecule is removed to form α, β-unsaturated acyl ACP.

vi) **Formation of Acyl ACP**

- The unsaturated acyl ACP is converted in the next step to a saturated acyl ACP by the enzyme **α,β-unsaturated acyl ACP reductase** using NADPH as the coenzyme.
- The resultant product contains two carbon atoms more than the starting material.
- Addition of subsequent acetyl units through malonyl ACP leads to the formation of 16-carbon palmitate.

Stoichiometry of fatty acid synthesis

The stoichiometry of the synthesis of palmitate is given below:

Acetyl CoA + 7 malonyl CoA + 14 NADPH + 20 H^+ ----------→

Palmitate + 7 CO_2 + 14 $NADP^+$ + 8 CoASH + 6 H_2O

The equation for the synthesis of the malonyl CoA used in the above reaction is

7 Acetyl CoA + 7 CO_2 + 7 ATP --------→ 7 malonyl CoA + 7ADP + 7 Pi + 14 H^+

The overall stoichiometry for the synthesis of palmitate is

8 Acetyl CoA + 7 ATP + 14 NADPH + 6H^+ --------→ Palmitate + 14 NADP + 8 CoASH + 6 H_2O + 7 ADP + 7 Pi

Fatty acid synthesis and degradation are reciprocally regulated so that both are not simultaneously active.

Elongation of fatty acids or synthesis of long chain fatty acids

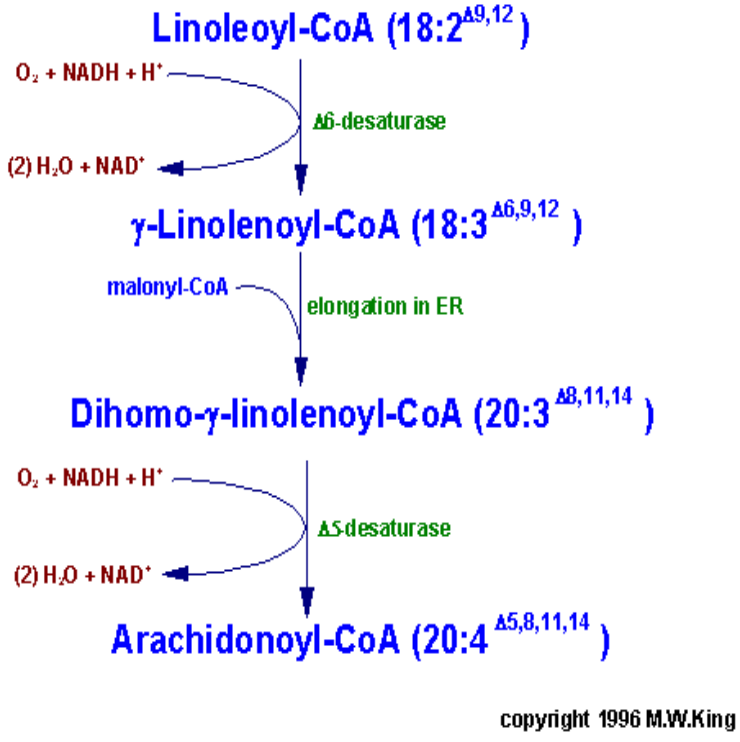
- Elongation by the **fatty acid synthase complex** stops upon formation of palmitate (16 C).

- Further elongation and the formation of double bonds are carried out by other enzyme systems**.**
- The major product of fatty acid biosynthesis is the 16-carbon fatty acid, palmitate.
- Additional enzymes are required to synthesise longer chain fatty acids.
- Chain elongation reactions occur both in **mitochondria and in microsomes.** Microsomes are small membrane-enclosed vesicles derived from the endoplasmic reticulum of cells.
- Mitochondria and microsomes carry out chain elongation by adding two-carbon units to fatty acids.
- The microsomal system has great physiological significance in that it provides the long chain fatty acids (18-24C) required for the **myelination of nerve cells in animal system.**
- Chain elongation occurs by a cycle **of condensation, reduction, dehydration followed by another reduction** that parallels cytosolic fatty acid biosynthesis.
- The more active elongation system adds **two carbons to palmitoyl-CoA to make it steroyl CoA.**
- The mechanism of elongation is identical with that known in the synthesis of palmitate except the enzyme systems and the acyl carrier protein.

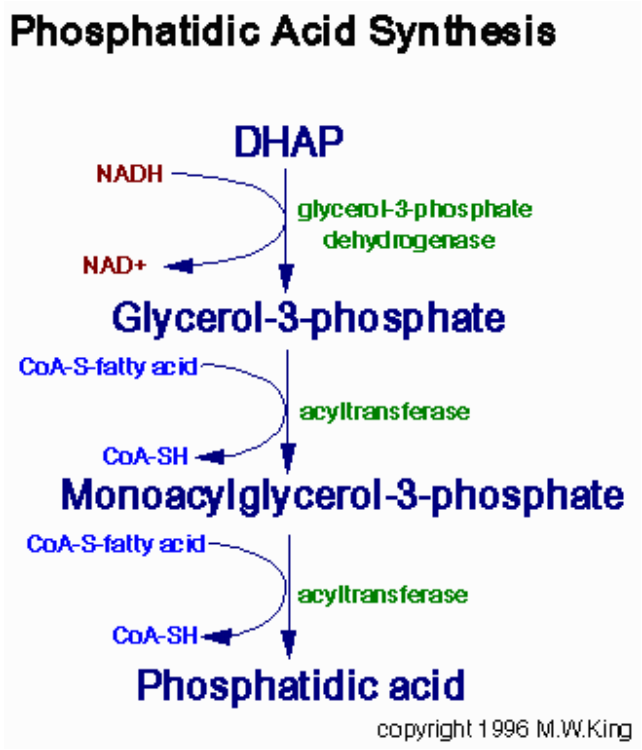
Biosynthesis of unsaturated fatty acids

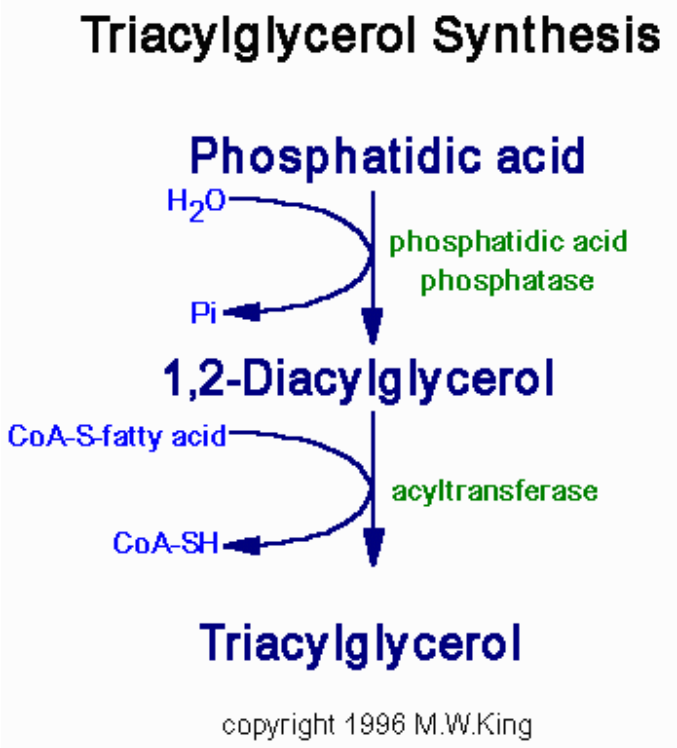
- Palmitate and stearate serve as precursors of the two most common monounsaturated fatty acids, **palmitoleate, 16:1, (Δ^9) and oleate, 18:1 (Δ^9)** respectively.
- Each of these fatty acids has a single double bond between C-9 and C-10.
- The **double bond is introduced into the fatty acid chain** by an oxidative reaction catalysed by **fatty acyl-CoA desaturase**, which is **NADPH-dependent enzyme.**

- The unsaturated fatty acids, linoleate, 18:2 ($\Delta^{9,12}$) and α-linolenate, 18:3 ($\Delta^{9,12,15}$) cannot be synthesised by mammals; but plants can synthesise both.
- The **desaturases** responsible for synthesis of both the above fatty acids are present in **endoplasmic reticulum of plants**.
- The plant desaturases oxidise phosphatidylcholine-bound oleate and produce polyunsaturated fatty acids and do not directly add double bonds to the fatty acids.
- Once ingested, the linoleate are readily converted to other **polyunsaturated fatty acids like γ-linolenate, arachidonic acid** etc. in animals and human beings.



Biosynthesis of Triacylglycerols





- Triacylglycerols are not synthesised by reversal of lipolysis.
- They are synthesisd by a different mechanism in which both glycerol and fatty acids are activated by ATP before they are incorporated into acylglycerols.

i) Activation of glycerol

- *Glycerol kinase* catalyses the activation of glycerol to glycerol 3-phosphate.
- If glycerol kinase is found in low quantity or absent, glycerol 3-phosphate will be formed from dihydroxyacetone phosphate obtained from glycolysis and this reaction is catalysed by the enzyme ***glycerol 3-phosphate dehydrogenase.***

ii) Activation of fatty acids

- Fatty acids are activated to acyl CoA by the enzyme acyl **CoA synthetase**, utilizing ATP and CoASH.
- Two molecules of acyl CoA combine with glycerol 3-phosphate to form 1,2-diacylglycerol phosphate.
- Formation of 1, 2-diacyl glycerol phosphate takes place in two stages, catalysed by **glycerol 3-phosphate acyl transferase** and then by **1-acyl glycerol 3- phosphate acyl transferase.**
- The phosphate group is removed from 1, 2-diacyl glycerol phosphate by **phosphatidate phosphatase** to form 1, 2-diacyl glycerol.
- Triacylglycerols are finally formed by esterification of one or more molecule of acyl CoA with the diacylglycerol.

Alternative pathway for triacylglycerol biosynthesis

- In this pathway, dihydroxyacetone phosphate from glycolysis is reduced by NADPH, acylated and converted to lysophosphatidate.
- This pathway accounts for less than 10% of total triacylglycerol synthesis.

Questions

Choose the correct answer

1. ---------- is converted to other PUFA like arachidonic acid

   a.Oleate b. Lipoleate c. Stearate d. Palmitate

   Ans: Lipoleate

2. Fatty acids are activated to acyl CoA by the enzyme

   a. Acyl CoA synthetase b.Acyl CoA Dhase c. Glycerol 3 –(p) dehydrogenase d. Lipase

   An: Acyl CoA synthetase

3. ---------- Catalyzes the activation of glycerol to glycerol-3-(p)

   a. Glycerol dehydrogenase b. Glycerolkinase c.Phosphoglycerokinase d.Phosphoglycerate mutase

   Ans: Glycerolkinase

True or False

4. Fatty acid biosynthesis occurs by reversal of β-oxidation pathway.

   Ans: True

5. Fatty acid degradation occurs in cytosol.

   Ans: False

Short notes

6. Biosynthesis of TAG
7. Formation of acyl ACP
8. Fatty acid synthase complex.

Lecture.22

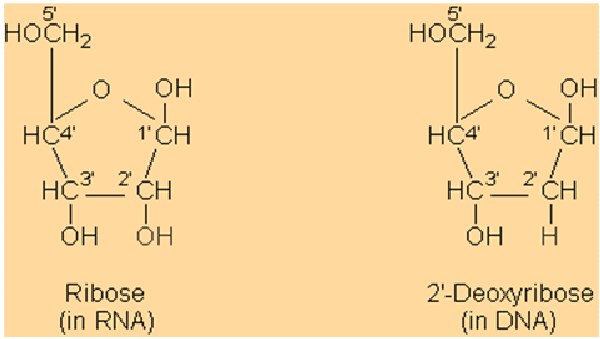
Nucleic acids: Structure and function

Macromolecular Structures: DNA

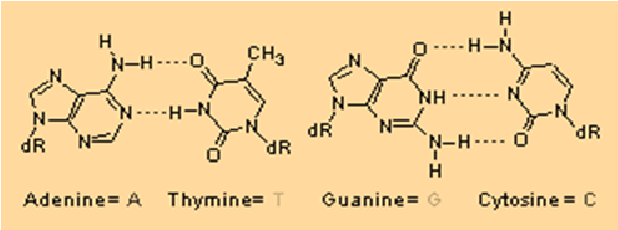
In general, a single cell contains ~ 10^4 – 10^5 different kinds of molecules. Roughly half of these molecules are small, whose molecular weights usually do not exceed several hundred (E.g. inorganic ions and organic compounds). The others are polymers that are so massive (molecular weights from 10^4 – 10^{12} Da) and are called as macromolecules. These molecules are of three classes: proteins, nucleic acids and polysaccharides, which are polymers of amino acids, nucleotides and sugars, respectively. There are also subclasses of these groups. E.g. glycoproteins (proteins carrying sugar groups), lipoproteins (proteins carrying lipids or fats), lipopolysaccharides etc. Knowledge of the properties of macromolecules is essential for understanding living process. In this lecture, the structure of nucleic acid will be studied in details because it is the molecule of live, which store and carry information of live which is the primary function of nucleic acid.

There are two kinds of nucleic acids- ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). A nucleic acid is a polynucleotide i.e. a polymer consisting of nucleotides. Each nucleotide has the three following components (Fig).

1. A cyclic five-carbon sugar: this is ribose, in the case of RNA and deoxyribose, in deoxyribonucleic acid DNA. The structure of ribose and 2'-deoxyribose differ only in the absence of a 2'-OH group in deoxyribose, *a difference that makes DNA chemically more stable than RNA.*



2. A purine or pyrimidine base attached to the 1'-carbon atom of the sugar by an N-glycosidic bond. The purines found in nucleic acids are adenine (A) and guanine (G) and the pyrimidines are cytosine (C), thymine (T) and Uracil (U). DNA and RNA both contain A, G and C. however, T is found only in DNA and U is found only in RNA. *There are exceptions to this rule- T is present in some tRNA molecules and there are few phages who's DNA exclusively contains U rather than T.*



3. A phosphate attached to the 5' carbon of the sugar by a phosphoester linkage. *This phosphate is responsible for the strong negative charge of both nucleotides and nucleic acids.*

A base linked to a sugar at position C1 is called as nucleoside. The sugar C1 carbon atom is joined to the N9 atom of purines and N1 atom of the pyrimidines by *β-N-glycosidic bond*. When a nucleoside linked with phosphate then it is called as nucleotide. The terminology used to describe nucleic acid components is listed in Table 1.

Table 1.Nucleotide nomenclature

Base	Nucleoside*	Nucleotides
Purine		
Adenine (A)	Adenosine (rA)	Adenylic acid or adenosine monophosphate (AMP)
	Deoxyadenosine (dA)	Deoxyadenylic acid or deoxyadenosine monophosphate (dAMP
Guanine (G)	Guanosine[$] (rG)	Guanylic acid or guanosine monophosphate (GMP)
	Deoxyguanosine (dG)	Deoxyguanylic acid or deoxyguanosine monophosphate (dGMP)

Pyrimidine		
Cytosine (C)	Cytidine (rC)	Cytidylic acid or cytidine monophosphate (CMP)
	Deoxycytidine (dC)	Deoxycytidylic acid or deoxycytidine monophosphate (dCMP)
Tymine (T)	Thymidine[@] (dT)	Thymidylic acid or thymidine monophosphate (TMP)
Uracil (U)	Uridine[#] (rU)	Uridylic acid or uridine monophosphate (UMP)

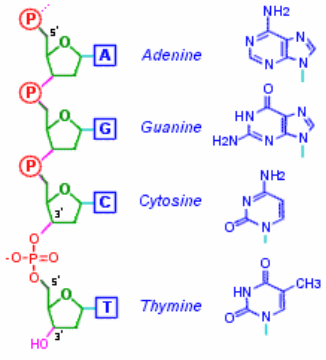
* Note that the names of purine nucleosides end in –osine and the names of pyrimidine nucleosides end in –idine. Note that in shorthand notation, nucleoside and nucleotide derivatives of deoxyribose are distinguished by the prefix'd' and 'r'. Only the second shorthand notation can discriminate between 5' and 3' phosphates, with 5' phosphate residues placed before the base (e.g. pA is adenosine-5'-monophosphate) and 3' phosphates placed after the base (e.g. Ap is adenosine-3'-monophosphate).

[$] Guanosine should not be confused with guanidine, which is not a nucleic acid base.

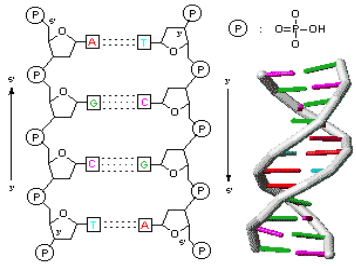
[@] Thymidine is the deoxy-form. The ribo form, ribosylthymine, is not generally found in nucleic acids

[#] Uridine is the ribo-form. Deoxyuridine is not commonly found, although deoxyuridylic acid is on the pathway for synthesis of thymidylic acid.

The nucleotides in the nucleic acids are covalently linked by a second *phosphoester bond* that joins the 5'phosphate of one nucleotide and 3' –OH group of the adjacent nucleotide (Fig). Thus, the phosphate is esterified to both 3' and 5' carbon atoms; this unit is often called a *phosphodiester group*. Thus a polynucleotide chain is formed.



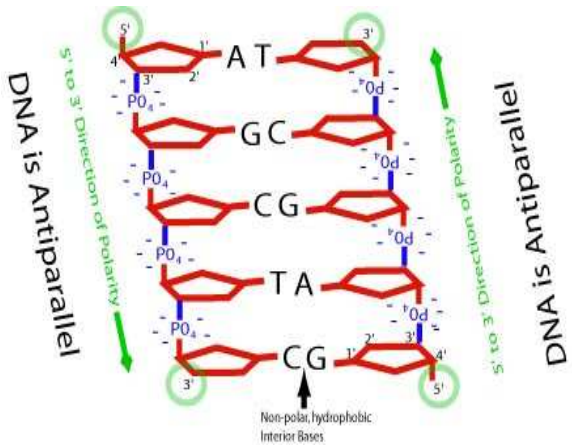
Two polynucleotides interact with one another and produce DNA double helix structure. This double helix structure was first proposed by James Watson and



Francis Crick in 1953 based on the X –ray diffraction studies of M. Wilkins and R. Franklin on DNA fibers (which revealed that the polynucleotide is helical and the bases of the nucleotides are stacked with their planes separated by a spacing of 3.4 A) and Chargraff's rule (the amounts of purine and pyrimidines present in the Organism are equal, i.e. A+T = G+C).

Watson and Crick combined chemical and physical data for DNA with a feature of the X-ray diffraction diagram that suggested that two helical strands are present in DNA and showed that the two strands are coiled about one another to form a double-stranded helix. The sugar phosphate backbones follow a helical path at the outer edge of the molecule and the bases are in a helical array in the central core. The bases of one strand are hydrogen bonded to those of other strand to form the purine-pyrimidine base pairs viz., A: T and G: C. Because each pair contains one two-ringed purine (A or G) and one single ringed pyrimidine (T or C, respectively), the length of each pair (in the sugar to sugar direction) is about the same and the helix can fit into a smooth cylinder.

The two polynucleotide strands of the DNA double helix are antiparellel i.e. the 3'-OH terminus of one strand is adjacent to the 5'-P (5' – phosphate) terminus of the other. The two bases in each base pair lie in the same plane and the plane of each pair is perpendicular to the helix axis. The base pairs are rotated 36° with respect to each adjacent pair, so there are 10 pairs per helical turn (Fig). The helix has two external helical grooves, a deep wide one (the major groove) and a shallow narrow one (the minor groove); both of these grooves are large enough to allow protein molecules to come in contact with the bases. Base pairing is one of the most important features of the DNA structure because it means that the base sequences of the two strands are complementary. In other words, purine in one strand is always pairs with pyrimidine in other strand i.e., if one strand has the base sequence AATGCT, the other strand has the sequence TTACGA, reading in the same direction. Specific pairing is achieved by reciprocal positioning of hydrogen bond acceptors and donors. Three



hydrogen bonds form in G:C base pairs and two in A:T (or A:U) base pairs. This has deep implications for the mechanism of DNAreplication because in this way, the replica of each strand is given the base sequence of its complementary strand. This form of DNA double helix, known as B-form, is prevalent *in vivo*. However, other forms of the helix (such as A-form, Z-form) with distinct structures also exist (Table 2). The A-form of DNA (which is prevalent *in vitro*) is less soluble than the B-form. That is why DNA, which is over dried during plasmid preparation, for example, is difficult to dissolve.

Table 2. Comparison of morphological features and helical parameters of the three major types of DNA helix.

Morphological Characteristics	Conformation		
	A	B	Z
Helical sense	Right	Right	Left
Pitch (base pairs per turn)	11	10	12
Major groove	Deep, narrow	Wide	Flat
Minor groove	Broad, shallow	Narrow	Narrow and very deep
Helix diameter	2.3 mm	1.9 mm	1.8 mm

Questions

1. The sugar residues are covalently joined by

a) 5’ -> 3’ phosphodiester bonds **b)** H_2 bonds **c)** phosphorylated ions **d)** covalent bonds

Ans: 5’ -> 3’ phosphodiester bonds

2. The bases in nucleic acids govern which one of the following process that underpins the essential biological process?

a) Replication **b)** recombination **c)** gene expression **d)** all the above

Ans: all the above

3. Nucleotide is made up of

a) Sugar **b)** phosphate **c)** nitrogenous base **d)** all the above

Ans: all the above

4. Which one of the following base is present both in DNA and RNA?

a) Adenine **b)** guanine **c)** cytosine **d)** all the above

Ans: all the above

5. The diameter of the double helix is

a) 20 Å **b)** 34 Å **c)** 3.4Å **d)** varies from one point to another

Ans: 20 Å

6. The number of bases in A form DNA per turn of the helix is

a) 20 **b)** 10 **c)** 34 **d)** 3.4

Ans: 10

7. Which one of the following phosphate moiety involved in 5' ->3' phosphodiester bond formation?

a) α **b)** β **c)** γ **d)** all the above

Ans: α

8. DNA or RNA polymerases are catalyze the 5' -> 3' phosphodiester bond formation and the byproduct of this reaction is

a) Pyrophosphate **b)** pyrophosphatase **c)** pyrosequences **d)** pyrates

Ans: pyrophosphate

9. Chargaff's rules state that

a) A=T **b)** G=C **c)** complementarity base paring **d)** all the above

Ans: all the above

10. The most stable nitrogenous bases in double helix DNA is

a) G: C **b)** A: T **c)** A: C **d)** T: G

Ans: G: C

11. Because of base interactions, the outside of the helix is not smooth and it is termed as

a) Major groove **b)** minor groove **c)** both a and b **d)** none of the above

Ans: both a and b

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Lecture.23

DNA Replication

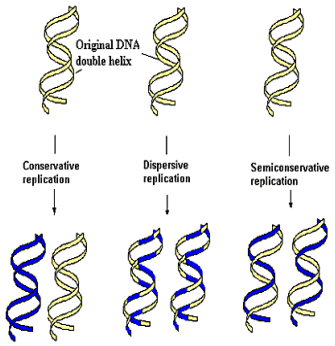
Genetic information is transferred from parent to progeny organisms by a faithful replication of the parental DNA molecules. At the biochemical level, replication is defined as a template-directed nucleic acid synthesis reaction where the template and nascent (growing) strand are the same type of nucleic acid. Replication is a polymerization reaction and can be divided into stages of initiation, elongation and termination.

Replication of dsDNA is a complicated process that is not completely understood due to the following facts:

1. A supply of energy is required to unwind the helix
2. The single strands resulting from the unwinding tend to form intrastrand base pairs
3. A single enzyme can catalyze only a limited number of physical and chemical reactions and many reactions are needed in replication.
4. Several safeguards have evolved that are designed both to prevent replication errors and to eliminate the rare errors that do occur
5. Both circularity and the enormous size of the DNA molecules impose geometric constraints on the replicative system and how this fit into the system has to be understood.

Models for the replication of DNA

The replication of cellular DNA was originally conceived as two models: conservative and dispersive. In conservative model, the parental DNA remains unchanged and gets passed to one daughter cell, whereas newly synthesized DNA gets passed to the other. But in dispersive replication, new DNA synthesis is interstitial (small openings), and each daughter cell receives a mixture of parental and newly synthesized DNA.



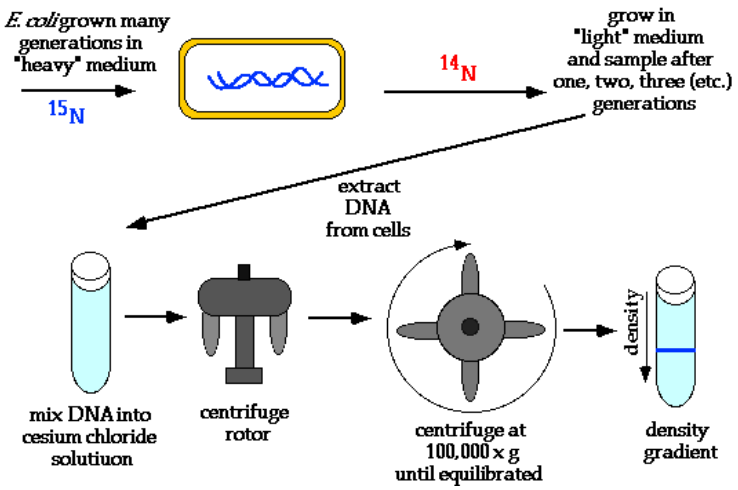
Possible Models of DNA Replication

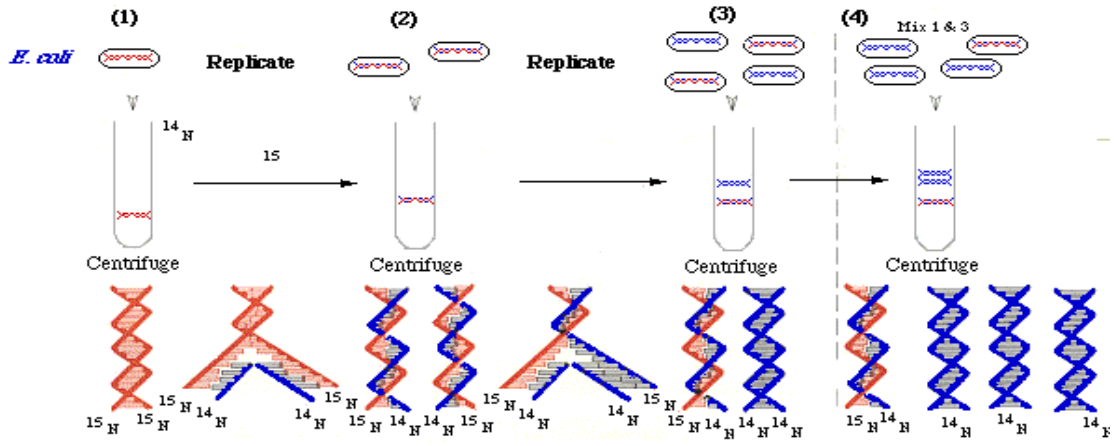
In a third, semi conservative model, proposed by Watson and Crick, the parental strands remain unchanged, but the duplex is separated into two halves. Each parental strand acts as a template for replication and the daughter duplexes have one parental strand and one daughter strand each.

The semi conservative model holds good for cellular DNA, but the single stranded genomes of viruses and some plasmids replicate conservatively - the structure of the single parental strand is conserved following replication.

Meselson-Stahl Experiment: Proof for Semiconservative DNA replication

In 1958, Mathew Meselson and Franklin Stahl showed that the replication of bacterial chromosomal DNA was semi conservative. *E. coli* were grown for many generations in a medium containing ^{15}N so that, their DNA became universally labeled with the isotope (heavy DNA). The cells were then shifted to a medium containing normal ^{14}N and DNA was isolated from cells after one and two rounds of replication. The DNA was analyzed by buoyant density centrifugation, which discriminates between heavy DNA and normal light DNA and intermediate DNA containing one heavy strand and one light strand. After one round of replication, the DNA was all of intermediate density and after two, there were equal amounts of intermediate density and light DNA (Fig). Thus, this experiment proved that cellular genomes replicate semi conservatively.





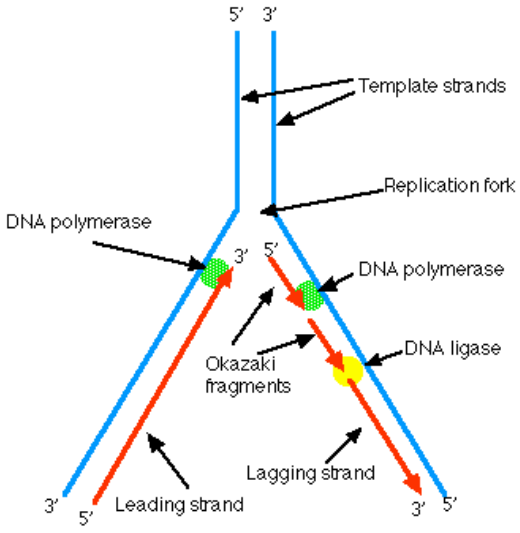
Semidiscontinuous replication

Watson and Crick's semi conservative model of DNA replication predicted the existence of a replication fork, a dynamic Y-shaped structure with a barrel composed of parental duplex DNA and arms composed of daughter duplex DNA, each daughter duplex consisting of one parental and one daughter strand (Fig). At the center of the fork, the parental duplex would be unwound and nucleotides would be added to the growing daughter strands. However, this model has a paradox, which can be summarized as follows:

1. cellular DNA replication is semi conservative
2. both daughter strands are extended simultaneously
3. the strands of the parental duplex are antiparellel
4. DNA polymerases extend DNA only in the 5' → 3' direction.

How can simultaneous 5'→ 3' elongation of both daughter strands occur at a replication fork when the parental templates have opposite polarity? This can be achieved by semidiscontinuous replication, where one strand is extended continuously and the other is synthesized discontinuously as a collection of short fragments. The mechanism of semidiscontinuous DNA replication can be formally expressed as leading strand – lagging strand model (Figure). The leading strand is the nascent strand, which is synthesized continuously in the direction of fork movement because its 3' end is exposed

to the DNA polymerase. The leading strand template is thus the *forward template*. The lagging strand is the nascent strand, which is synthesized discontinuously in the opposite direction to the fork movement because its 5' end, the end.

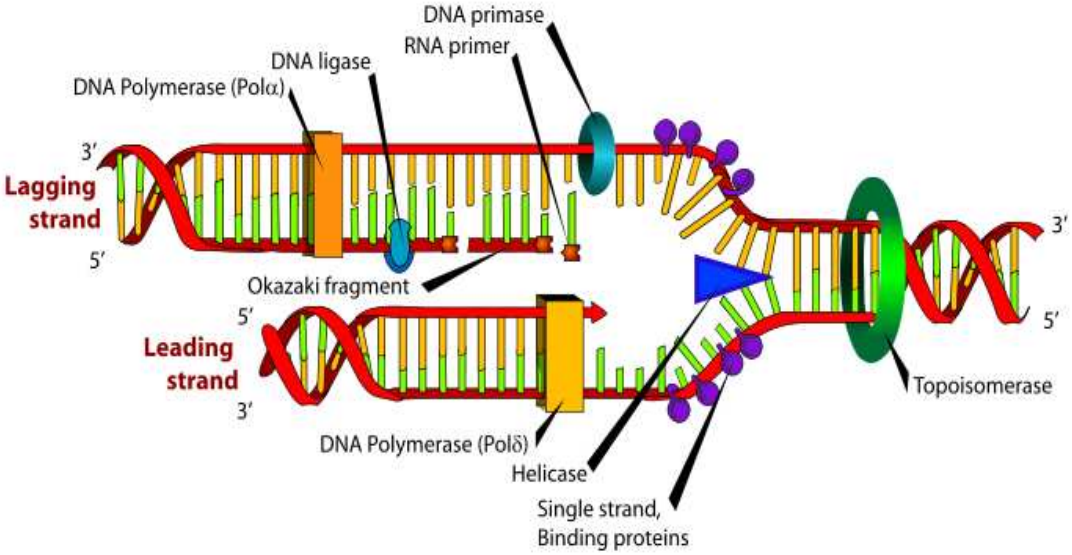


Leading strand – lagging strand model cannot be extended, is exposed to the DNA polymerase. The lagging strand is thus the *retrograde template.* The mechanism can be summarized as follows: as the replication fork moves forward and the leading strand is extended, a portion of retrograde template is exposed. DNA polymerase can then synthesize a small fragment of DNA, an *Okazaki fragment*, by moving backwards over the template in relation to the fork progression. The lagging strand is so called because the leading strand must be synthesized first to uncover the corresponding portion of lagging strand template. The enzyme dissociates from the template when it reaches the previously synthesized Okazaki fragment, by which time a further portion of retrograde template has been exposed. The enzyme can then reinitiate and synthesize a new Okazaki fragment. By repeating this back-stitching process over and over, the lagging strand would appear to grow in the 3'→5' direction.

Mechanism of DNA replication

Initiation

Most organisms contain one or more enzymes called topoisomerases, which can produce variety of topological changes in DNA. The brief outline of replication mechanism in *E. coli* is described here. An enzyme called as helicase binds with single strand binding (SSB) protein along with DnaB protein and unwinds the helix. The unpaired bases are coated with SSB. DNA gyrase (Eco topoisomerase II) has the ability to produce negative superhelicity generated during replication. That is, positive



superhelicity is removed by gyrase introducing negative twists by binding ahead of the advancing replication fork.

Elongation

The leading strand advances along one parental strand by nucleotide addition catalyzed by the pol III holoenzyme (DNA polymerase III). The term holoenzyme refers to an enzyme that contains several different subunits and retains some activity even when one or more subunits are missing. In *E. coli*, two types of DNA polymerases exist viz., pol I and pol III. They are able to synthesize DNA from four precursor molecules, four-deoxynucleoside 5'-triphosphates (dNTPs viz., dATP, dGTP, dTTP and dCTP), as long as a DNA molecule to be copied (template DNA) is provided. Neither 5'monophosphates nor 5' diphosphates, nor 3'-mono, di, tri-phosphates can be polymerased; only the

5’triphosphates are substrate for the polymerization reaction. In addition they require nucleic acid fragment to initiate the polymerization. The overall chemical reaction catalyzed by the DNA polymerase is:

Poly (nucleotide)$_n$-3’-OH + dNTP $\Leftrightarrow$ Poly (nucleotide)$_{n+1}$-3’-OH + PP$_i$

The polymerase also catalyzes depolymerization. In order to drive the reaction to the right, pyrophosphate must be removed, and this is done by a potent pyrophosphatase, a widely distributed enzyme that breaks down pyrophosphate to inorganic phosphate.

In addition, polymerization occurs only is the presence of primer- that is an oligonucleotide hydrogen bonded to the template strand and whose terminal 3’-OH group is available for reaction. Because polymerization consists of a reaction between a 3’OH group at the end of the growing strand and an incoming nucleoside-5’ triphosphate. When the nucleotide is added it supplies another free 3’-OH group.

The primer for both leading and lagging strand synthesis is a short RNA oligonucleotide that consists of 1 to 60 bases; the exact number depends on the particular organism. This RNA primer is synthesized by copying a particular base sequence from one DNA strand and differs from a typical RNA molecule, in that after its synthesis the primer remains hydrogen bonded to the DNA template. In bacteria two different enzymes are known that synthesize primer RNA molecules – RNA polymerase and Primase. The DnaB protein complex moves along the other parental strand, prepriming it so that primase will synthesis a primer RNA. Pol III holoenzyme adds nucleotides to the primer, thereby synthesizing a precursor fragment. This synthesis continues up to the primer of the preceding precursor fragment.

Apart from this function, DNA polymerases also has 3’$\rightarrow$5’ exonuclease, 5’$\rightarrow$3’ exonuclease and endonuclease activity and so they can perform nick translation and strand displacement. By nick translation the RNA is removed and replaced by DNA. Once the RNA is gone, DNA ligase seals the nick, thereby joining the precursor fragment to the lagging strand. Pol II moves back along the DNA (in the direction of advancement of the fork) until it encounters the next primer and the process continue again and again.

Since each strand has 5'-P terminus and 3'-OH terminus, strand growth is said to proceed in the 5'→3' direction (Fig). The advance of the replication fork continues until replication is completed. An unsolved question is how the rates of growth of the leading and lagging strands are coordinated.

Termination

In a unidirectionally replicating molecule, replication terminates at the origin. In bidirectionally replicating molecule, it may be of two types: 1. there might be definite termination sequence. 2. Two growing points collide and termination occurs where ever the collision point happens to be.

Replication in Eukaryotes

The complete mechanism of initiation, elongation and termination of linear DNA molecule and chromatin replication has not yet been elucidated. However, it is believed that there might be multiple replication forks exist during replication. Similarly different isoforms of DNA polymerases have been identified in eukaryotes with specific functions.

Fidelity of DNA replication

There is no single molecule whose integrity is as vital to the cell as DNA. Thus, in the course of hundreds of millions of years there have evolved efficient systems for correcting the occasional mistakes that occur during replication. DNA repair/damage can occur as the result of exposure to environmental stimuli such as alkylating chemicals or ultraviolet or radioactive irradiation and free radicals generated spontaneously in the oxidizing environment of the cell. These phenomena can, and do, lead to the introduction of mutations in the coding capacity of the DNA. Mutations in DNA can also, but rarely, arise from the spontaneous tautomerization of the bases (the rare imino form of adenine can form a stable hydrogen bond with cytosine and the enol form of thymine can pair with guanine).

E. coli cells possess at least five distinct mechanisms for the repair of defects in DNA: 1) light-dependent repair or photoreactivation, 2) excision repair, 3) mismatch

repair, 4) post-replication repair and 5) error-prone repair. Mammals seem to possess all of the repair mechanisms found in *E. coli* except photoreactivation.

Questions

1. Who has shown that replication is semi-conservative event?

a) Mathew Meselson and Franklin Stahl b) Watson and Crick

c) Delbruck and Korenberg d) none of the above

Ans: Mathew Meselson and Franklin Stahl

2. The enzyme which unwinds DNA ahead of replication fork is

a) DNA helicase b) DNA ligase c) DNA polymerase d) DNA primase

Ans: DNA helicase

3. *Taq* DNA polymerase, widely used in PCR, is homologous to *E. coli* pol I but lacks

a) 3'→5' exonuclease activity b) 5'→3' exonuclease activity

c) 5'→3' polymerase activity d) 3'→5' polymerase activity.

Ans: 3'→5' exonuclease activity

4. During replication, the functional complex primosome is formed by

a) primase b) helicase c) both a and b d) α –polymerase.

Ans: both a and b

5. Enzymes which digest nucleic acid by hydrolyzing phosphodiester bonds are called as

a) nucleases b) deoxyribo nucleases (if substrate is DNA)

c) ribonucleases (if substrate is RNA) d) all the above

Ans: all the above

6. Which of the following is true?

a) DNA polymerase recognizes 'ori' in parental DNA and unwinds the double helix at these sites so that DNA replication can occur.

b) Helicases recognize replication fork and unwinds the double helix at replication fork so that DNA replication can occur

c) Helicases recognize origin of replication in parental DNA and unwinds the double helix at these sites so that DNA replication can occur

d) None of the above

Ans: Helicases recognize origin of replication in parental DNA and unwinds the double helix at these sites so that DNA replication can occur

7. If the parental DNA strand, 3' GGCATATTCGCTGCAGT 5', is used as a template DNA strand the newly synthesized, antiparallel strand would be as follows

8. The sequence of one strand of DNA is 5'-AGTCGACGA-3'. What would be the 5' to 3' sequence of the complementary strand?

a) 5' – TCAGCTGCT – 3' b) 5' – TCGTCGACT – 3'

c) 5' – AGTCGACGA - 3' d) too little information to predict

Ans: 5' – TCGTCGACT – 3'

9. In Eukaryotes, the DNA replication occurs during

a) S phase b) G1 phase c) G2 phase d) all phases

Ans: S phase

10. DNA ligase

a) Unwinds the helical DNA by breaking the hydrogen bonds between complementary bases

b) Adds DNA nucleotides to the RNA primer

c) Links the DNA fragments of the lagging strand together

d) Synthesizes a short RNA primer at the beginning of each origin of replication

Ans: Links the DNA fragments of the lagging strand together

11. The 5' end of the DNA is the one with

a) the terminal phosphate group on the 5' carbon of the deoxyribose

b) a terminal hydroxyl (OH) group on the deoxyribose of the 3' carbon of the deoxyribose

c) a terminal hydroxyl (OH) group on the deoxyribose of the 5' carbon of the deoxyribose

d) None of the above

Ans: the terminal phosphate group on the 5' carbon of the deoxyribose

12. _____ is one correct reason for why Okazaki fragments are created during lagging strand DNA synthesis

a) DNA is only polymerized in the 3' to 5' direction.

b) DNA polymerase requires RNA priming

c) DNA is only polymerized in the 5' to 3' direction

d) DNA polymerase requires a 5' -0H on the growing polymer

Ans: DNA is only polymerized in the 5' to 3' direction

13.At the completion of DNA replication, each newly synthesized DNA strand is

a) Identical in sequence to the strand opposite which it was synthesized
b) Complementary in sequence to the strand opposite which it was synthesized
c) A hybrid stand consisting both DNA and RNA strand
d) Oriented in the same direction as the strand which it was synthesized

Ans: Complementary in sequence to the strand opposite which it was synthesized

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Lecture.24
Transcription

The transfer of genetic information from DNA to RNA molecules and then from RNA to protein molecules accomplishes Gene expression. RNA molecules are synthesized by using a portion of one strand of DNA as a template in a polymerization reaction that is catalyzed by enzymes called RNA polymerases. The process by which RNA molecules are initiated, elongated and terminated is called transcription. Two aspects of transcription must be considered – 1. The enzymology and 2. The signals that determine where on a DNA molecule transcription begins and stops.

Gene

The term gene was coined by Wilhelm Johansen in 1909 to describe a heritable factor responsible for the transmission and expression of a given biological character. In 1911, T. H. Morgan showed that genes were located on chromosomes and were physically linked together and in 1944, O. Avery and his colleagues shown that DNA was the genetic material. Thus, a simple picture of a gene evolved - a length of DNA in a chromosome, which encoded the information for a protein. At any given locus, the DNA which is transcribed can be termed a transcription unit. In prokaryotes, a transcription unit may consists of several genes (constituting an operon) whereas in eukaryotes, transcription units are almost always equivalent to a single gene.

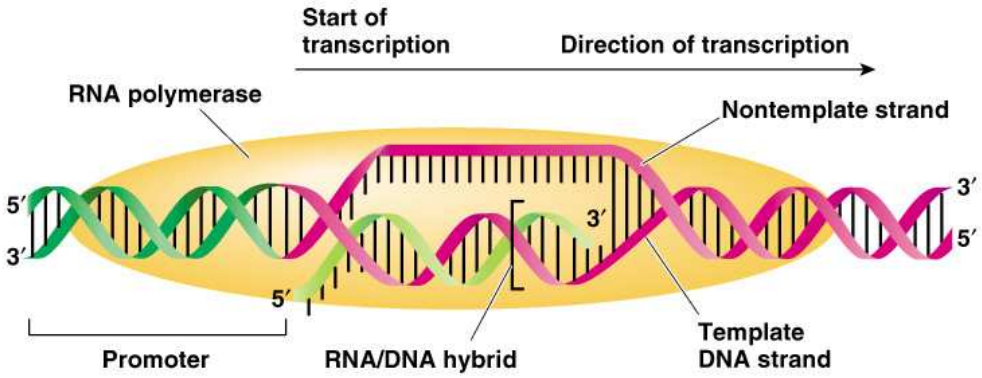
In short, Gene (Cistron) is the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer sequence) as well as intervening sequences (introns) between individual coding segments (exons).

Basic features of RNA synthesis

Thus, a gene, which code the genetic information first transcribed as RNA. The essential chemical characteristics of the synthesis of RNA are following:

1. The precursors in the synthesis of RNA are the four ribonucleoside 5’ – triphosphates (NTP) ATP, GTP, CTP and UTP.

2. In the polymerization reaction a 3'OH group of one nucleotide reacts with the 5' – triphosphate of a second nucleotide; pyrophosphate is removed and a phosphodiester bond results by the activity of RNA polymerase.
3. The sequence of bases in an RNA molecule is determined by the base sequence of the DNA. Each base added to the growing end of the RNA chain is chosen by its ability to base – pair with the DNA strand used as a template; thus the bases C, T, G and A in a DNA strand cause G, A, C and U, respectively, to appear in the newly synthesized RNA molecule.
4. The DNA molecule being transcribed is double stranded, yet in any particular region only one strand serves as a template.



5. RNA chain grows in the 5'→3' direction (antiparellel) as that of DNA synthesis.
6. RNA polymerases, in contrast with DNA polymerases, are able to initiate chain growth; that is, no primer is needed.
7. Only ribonucleoside 5'- triphosphates participate in RNA synthesis and the first base to be laid down in the initiation event is a triphosphate (Fig).

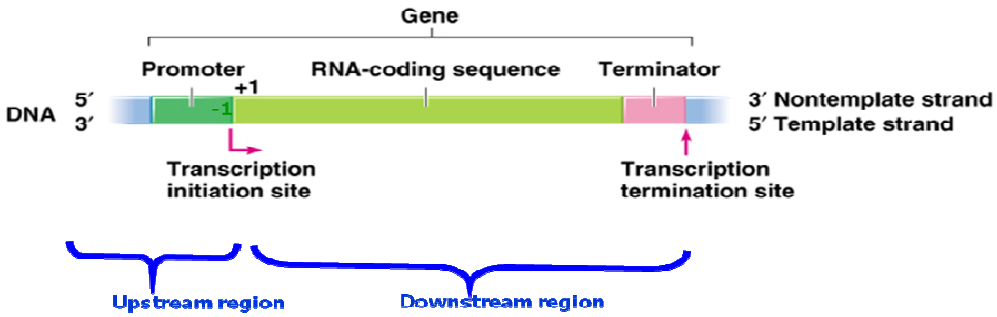
The synthesis of RNA consists of four discrete stages: 1. binding of RNA polymerase to a template at a specific site 2. initiation 3. chain elongation 4. Chain termination and release.

Binding of RNA polymerase

E. coli RNA polymerase consists of five subunits – two identical α subunits and one each types of β, β' and σ. The σ subunit dissociates from the enzyme during the elongation stage of RNA polymerization. The term core enzyme is used to describe the

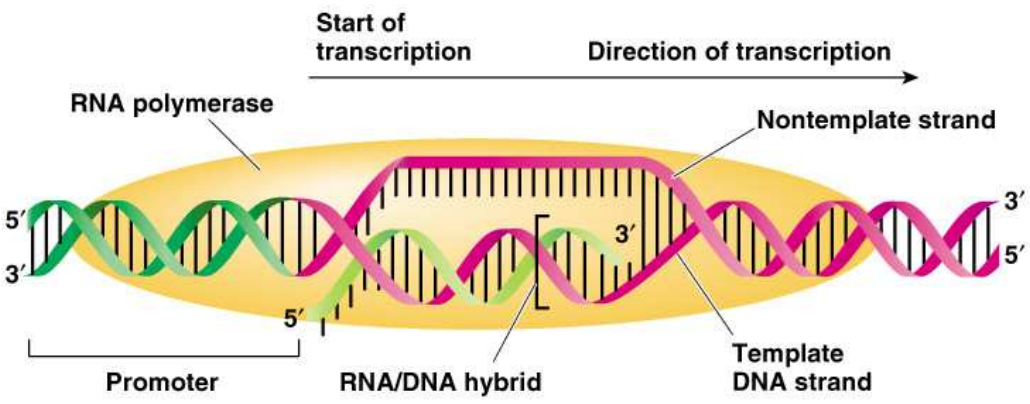
σ-free unit, $\alpha_2\beta\beta$. The complete enzyme, $\alpha_2\beta\beta\sigma$ is called the holoenzyme. RNA polymerase is sufficiently large that it can come into contact with many DNA bases simultaneously. The first step in transcription is the binding of RNA polymerase to a DNA molecule. Binding occurs in particular regions called promoters, which are sequences in which several interactions occur.

Initiation: RNA polymerase recognizes the promoter



Several events occur at a promoter: RNA polymerase recognize a specific DNA sequence, attach in a proper conformation, locally open the DNA strands in order to gain access to the bases to be copied and then initiate synthesis. These events are guided by the base sequence of the DNA, the polymerase σ subunit (without which the promoter is not recognized).

The specific binding region in DNA is in a region 5 –10 bases prior to the left of the first base copied into mRNA. This region is called as Pribnow box. All sequences found in Pribnow boxes are considered to be variants of a basic sequence TATAAT, hence they are called as TATA box. The first base transcribed was chosen as a reference point and numbered +1. The direction of transcription was called downstream; all upstream bases, which are not transcribed, were given negative numbers starting from the reference. Thus, the Pribnow box is enclosed between –21 and –4 depending on the particular promoter.



There is a second important region, to the left of the Pribnow box, whose sequences in different promoters have common features. This six base sequence which is called as the "–35 sequence "has a consensus TTGACA, may be initial site of binding of the enzyme, when the sequence is present.

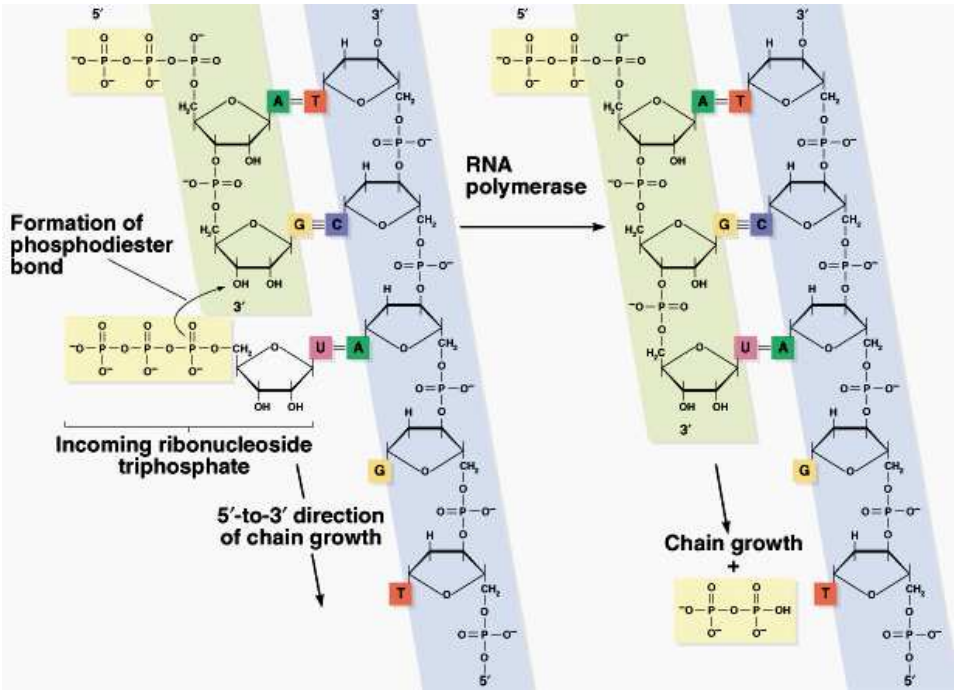
The Pribnow box is thought to orient RNA polymerase, such that synthesis proceeds from left to right and to be the region at which the double helix opens to form the open- promoter complex. This highly stable complex is the active intermediate in chain initiation. The DNA double helix in an open-promoter complex is locally unwound, starting about 10 bp from the left end of Pribnow box and extending about 20 bp past the position of the first transcribed base. It seens that RNA polymerase itself induces this unwinding and undergoes a conformational change itself in so doing. This melting is necessary for pairing of incoming ribonucleotides.

2. RNA chain initiation

Once the open promoter complex has formed, RNA polymerase is ready to initiate synthesis. RNA polymerase contains two nucleotide binding sites called the initiation site and the elongation site. The initiation site primarily binds to purine triphosphates, ATP or GTP. ATP is usually the first nucleotide in the chain. The initiating nucleoside triphosphate binds to the enzyme in the open-promoter complex and forma a hydrogen bond with the complementary DNA base. The elongation site (also called the catalytic site) is the filled with a nucleoside triphosphate that is selected by its ability to hydrogen-bond with the next base in the DNA strand. The two nucleotides are then joined together, the first base is released from the initiation site and initiation is complete. In some way, the details of which are not understood, the RNA polymerase and the template strand move relative to each other, so the binding sites and the catalytic sites are shifted by exactly one nucleotide. The drug, rifampicin is useful in studying initiation. It binds to the β subunit of RNA polymerase blocking the transition from the chain initiation phase to the elongation phase.

Chain elongation

After several nucleotides (~ 8) are added to the growing chain, RNA polymerase undergoes a conformational changes and loses the σ subunit. Thus now this process enters into elongation phase, and most elongation is carried out by the core enzyme of RNA polymerase. The core enzyme moves along the DNA, binding a nucleoside triphosphate that can pair with the next DNA base and opening the DNA helix as it moves. The DNA helix resumes its original shape as synthesis proceeds. The newly synthesized RNA is released from its hydrogen bonds with the DNA as the helix re-forms.

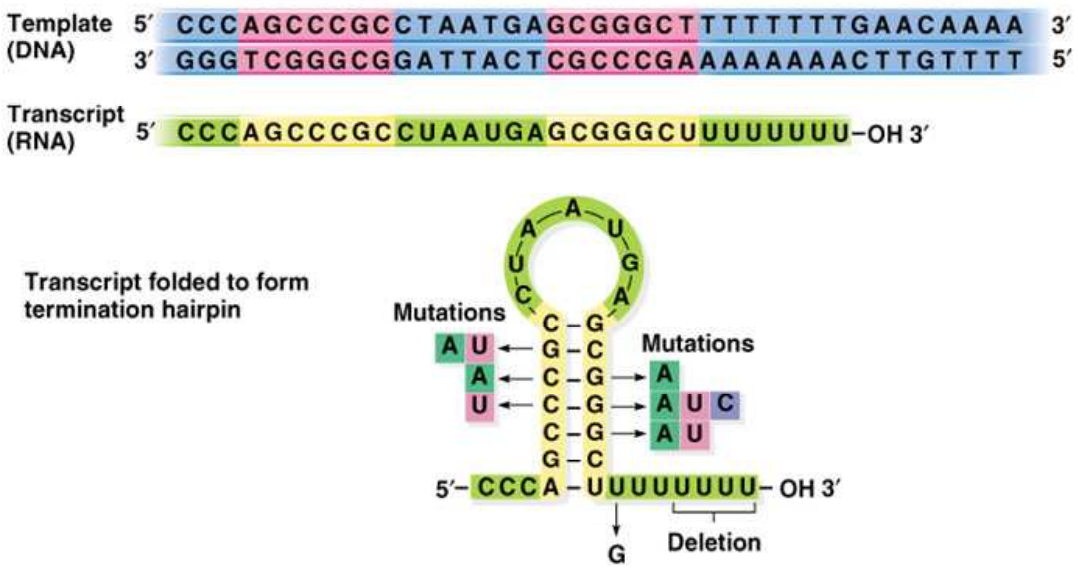


Termination and release of new RNA

Termination of RNA synthesis occurs at specific base sequences within the DNA molecule. These sequences are of two types, simple terminators and those that require auxiliary termination factors. At a particular sequence, if there is an inverted repeat containing a central nonrepeating segment, ie., the sequence in one DNA strand would read like TATAT- NNN – ATATA, then the RNA transcribed from this strand form a

intrastrand base paring and leads to stem and loop structure. Termination occurs at this hairpin region for unknown reasons.

Alternatively, termination also occurs due to auxiliary termination protein called – Rho protein. It binds tightly with RNA which has segment that are rich in C (especially repeating C's). The binding helps the protein to acquire a powerful ATP cleaving activity that is essential to its action in termination. This is because, nucleoside triphosphates cannot reach RNA polymerase since they are degraded by Rho protein. Significant differences exist between the structures and modes of synthesis of the RNA molecules of prokaryotes and eukaryotes, though the basic mechanisms of their functions are nearly the same.



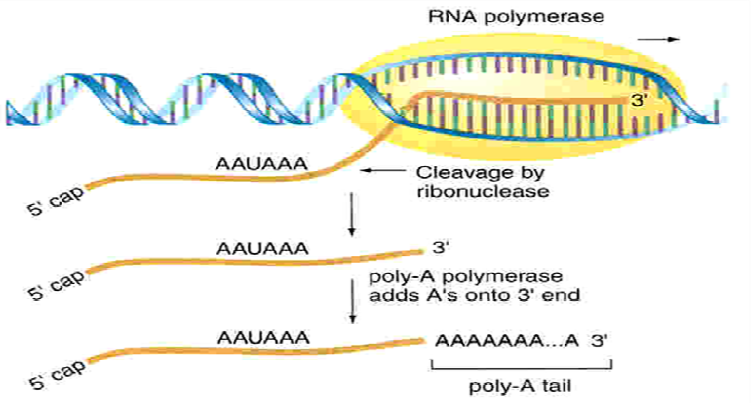
Classes of RNA molecules

There are three major classes of RNA molecules – messenger RNA (mRNA; an informational molecule), ribosomal RNA (rRNA; a structural molecule) and transfer RNA (tRNA; a structural and informational molecule).

mRNA

The base sequence of a DNA molecule determines the amino acid sequence of every polypeptide chain in cell, though amino acids have no affinity for DNA. Thus, instead of direct pairing between amino acids and DNA, a multistep process is used in

which the information contained in the DNA is converted to a form in which amino acids can be arranged in an order determined by the DNA base sequence. This process begins with the transcription of the base sequence of one of the DNA strands (the coding strand) into the base sequence of an RNA molecule (mRNA). The protein synthesizing machinery of the cell obtain the information, i.e., the amino acid sequence of a particular protein to be synthesized, from this RNA molecule. The nucleotide sequence of the mRNA is then read in groups of three bases (a group of three is called as Codon) from a start codon to stop codon, with each codon corresponding either to one amino acid or a stop signal.



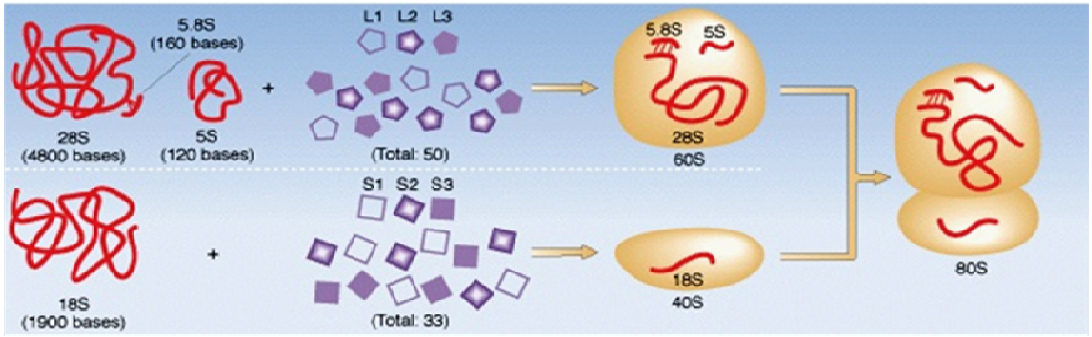
A DNA segment corresponding to one polypeptide chain plus the translational start and stop signals for protein synthesis is called a cistron and an mRNA encoding a single polypeptide is called monocistronic mRNA. In prokaryotes, it is very common for an mRNA molecule to encode several different polypeptide chains; in this case it is called as polycistronic mRNA. The segment of RNA corresponding to a DNA cistron is often called a reading frame, since the protein synthesizing system reads it.

In addition to reading frames and start and stop sequences for translation, other regions in mRNA are significant. Translation of an mRNA molecule rarely starts exactly at one end of the RNA and proceeds to the other end; instead, initiation of synthesis of the first polypeptide chain of a polycistronic mRNA may begin hundreds of nucleotides from the 5'- P terminus of the RNA. The section of untranslated RNA before the coding regions is called a leader. In some cases, the leader contains a regulatory region.

Untranslated sequences usually found at both the 5'-P and 3'-OH termini and a polycistronic mRNA molecule typically contain intercistronic sequences (spacers) usually tens of bases long. An important characteristic of prokaryotic mRNA is that its lifetime is short (only few minutes) compared to other types of RNA molecules.

Ribosomal RNA and transfer RNA

During the protein synthesis, genetic information is supplied by mRNA. Amino acids do not line up against the mRNA template independently during protein synthesis but are aligned by means of a set of about 50 adaptor RNA molecules called transfer RNA (tRNA) and this is occurred on the surface of an RNA-containing protein particle called as ribosome. These particles consist of several classes of ribosomal RNA (rRNA) and ribosomal proteins, which are stable molecules and having various functions. Whereas the transfer RNA molecule exist in the cell, has a capacity of 'reading' three adjacent mRNA bases and placing corresponding amino acid at a site on the ribosome at which a peptide bond is formed with an adjacent amino acid. Neither rRNA nor tRNA is translated into polypeptide chain.



Ribosome structure (eukaryotes)

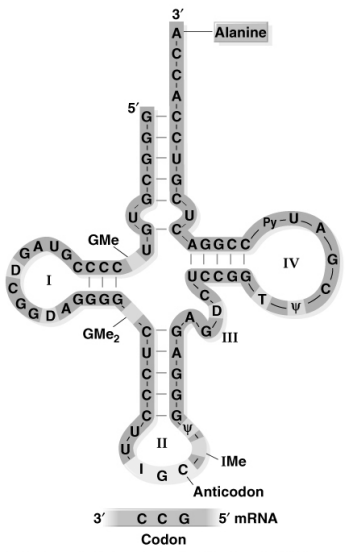
23S & 5S rRNA 34 proteins (L1-L31) 30S& 50S 70S (prokaryotes)
16S rRNA 21 proteins (S1-S21)

The synthesis of both rRNA and tRNA molecules is initiated at a promoter and completed at terminators; in this respect, their synthesis is no different from that of mRNA. However, the following three properties of these molecules indicate that neither rRNA nor tRNA molecules are the immediate products of transcription (called as primary transcripts):

1. The molecules are terminated by a 5' monophosphate rather than the expected triphosphate found at the ends of all primary transcripts.
2. Both rRNA and tRNA molecules are much smaller than the primary transcripts.
3. All tRNA molecules contain bases other than A, G, C and U and these unusual bases are not present in the original transcript.

All of these molecular changes are made after transcription by processes collectively called as posttranscriptional modification or more commonly, processing.

All ribosomes comprise two dissimilar sized subunits, the large and small subunits. Each subunits consists of several rRNA and numerous ribosomal proteins (r-proteins). In *E. coli*, the 70S ribosome is composed of a small 30S subunit and a large 50S subunit. The small subunit comprises 21 different proteins (named S1-S21) and the 16S RNA. The large subunit comprises 34 proteins (named L1-L34) and the 23S and 5S rRNAs. Some proteins are common to both subunits (e.g. L6, S20). Eukaryote ribosomes are larger (80S) and contain more components. The small (40S) subunit comprises 33 proteins and the 18S rRNA whilst the large (60S) subunit contains 50 proteins and three rRNAs of 28S, 5.8S and 5S. The spatial organization of the ribosome is complex. rRNA makes up 60-65 % pf the total mass and is essential for structural integrity and function, adopting complex tertiary and quaternary conformations by intra and inter molecular base pairing.



The tRNAs are relatively homogeneous family of RNA molecules, usually 75-100 nucleotides in length, which are extensively processed during their production. They possess a characteristic secondary and tertiary structure (Figure), most importantly the acceptor stem (to which the amino acid binds) and the anticodon loop (which carries the three nucleotide anticodon that forms complementary base pairs with codons in the mRNA). Bacterial cells contain up to 35 different tRNAs and eukaryotic cells up to 50. This number is lower than the number of possible codons in the genetic code, but greater than the number of amino acids specified by the code. This indicates that individual

tRNAs can recognize more than one codon (called as wobble pairing), but that different tRNAs may be charged with the same amino acids (these are called as isoaccepting tRNAs). The tRNAs are charged (conjugated to their corresponding amino acids) by enzymes termed amino acyl tRNA synthetases. There is one enzyme for each amino acid and therefore each synthetase recognizes all its cognate isoaccepting tRNAs.

Genetic Code

The genetic code is the collection of base sequences (called as codons) that corresponds to each amino acid and to stop signals for translation. Since there are 20 amino acids, there must be more than 20 codons to include signals for starting and stopping the synthesis of particular protein molecules. If one assumes that all codons have the same number of bases, then each codon must contain at least three bases. Because: A single base cannot be a codon since there are 20 amino acids and only 4 bases. Pairs of bases also cannot serve as codons because there are only $4^2 = 16$ possible pairs of four bases. Triplets of bases are possible because there are $4^3 = 64$ triplets, which is more than adequate. In many cases, several codons designate the same amino acid- that is the code is redundant or degenerate. In the in vitro system, protein synthesis can start at any base. However, in vivo it starts only at AUG codon. Similarly it stops at either UAA, UGA or UAG.

Tabel 1. The universal genetic code

First letter	Second letter: U	Second letter: C	Second letter: A	Second letter: G	Third letter
U	UUU Phe (F)	UCU Ser (S)	UAU Tyr (Y)	UGU Cys (C)	U
	UUC Phe (F)	UCC Ser (S)	UAC Tyr (Y)	UGC Cys (C)	C
	UUA Leu (L)	UCA Ser (S)	UAA Stop	UGA Stop	A
	UUG Leu (L)	UCG Ser (S)	UAG Stop	UGG Trp (W)	G
C	CUU Leu (L)	CCU Pro (P)	CAU His (H)	CGU Arg (R)	U
	CUC Leu (L)	CCC Pro (P)	CAC His (H)	CGC Arg (R)	C
	CUA Leu (L)	CCA Pro (P)	CAA Gln (Q)	CGA Arg (R)	A
	CUG Leu (L)	CCG Pro (P)	CAG Gln (Q)	CGG Arg (R)	G
A	AUU Ile (I)	ACU Thr (T)	AAU Asn (N)	AGU Ser (S)	U
	AUC Ile (I)	ACC Thr (T)	AAC Asn (N)	AGC Ser (S)	C
	AUA Ile (I)	ACA Thr (T)	AAA Lys (K)	AGA Arg (R)	A
	AUG Met (M)	ACG Thr (T)	AAG Lys (K)	AGG Arg (R)	G
G	GUU Val (V)	GCU Ala (A)	GAU Asp (D)	GGU Gly (G)	U
	GUC Val (V)	GCC Ala (A)	GAC Asp (D)	GGC Gly (G)	C
	GUA Val (V)	GCA Ala (A)	GAA Glu (E)	GGA Gly (G)	A
	GUG Val (V)	GCG Ala (A)	GAG Glu (E)	GGG Gly (G)	G

Questions

1. Transcription, the synthesis of RNA using DNA as template, is required for

a) Gene expression b) DNA replication c) Translation d) all the above

Ans: all the above

2. Transcription is asymmetric *i.e.*,

a) both DNA strands are used as template b) only one of the strand of DNA is used as template c) combinations of different parts of DNA is used as template d) all the above

Ans: one of the strand of DNA is used as template

3. The nascent RNA strand synthesized as continuous strand during transcription and it is analogous to

a) Lagging strand b) leading strand c) antiparallel strands d) all the above

Ans: leading strand

4. The nucleotide immediately preceding the transcription unit on the DNA strand is defined as position

a) +1 b) 0 c) -1 d) 5' UTR

Ans: -1

5. RNA polymerases

a) Require RNA primers b) do not require primer and initiate strand synthesis *de novo*

c) do not proofread their transcripts d) both b and c

Ans: both b and c

6. Transcription factors recognize and binds to

a) Silencer b) enhancer c) promoter d) all the above

Ans: all the above

7. Roger Kornberg got Noble prize for the year 2006 for his work on

a) RNA Polymerase b) Histone c) DNA Polymerase d) Ribosome

Ans: RNA Polymerase

8. Which of the following features would you NOT expect to find in heterogeneous nuclear RNA (hnRNA)?

a) Intron b) polycistronic coding c) polyadenylation at 3'-end d) 5-' cap structure

Ans: polycistronic coding

9. The non-coding DNA that occurs within eukaryotic gene is referred to as

a) Spacer DNA b) Promoter c) terminator d) Intron DNA

Ans: Intron DNA

10. Which of the following statements about introns is incorrect? Introns are

a) Found in most eukaryotic genes and not translated during protein synthesis

b) Removed during RNA processing

c) Normally not transcribed

d) Responsible for the fact that the most eukaryotic mRNAs are much shorter than the genes from which they are derived.

Ans: Normally not transcribed

11. DNA is double stranded. One strand is called the coding strand and the other the non-coding strand. The non-coding strand is used as the template to make mRNA. The relationship between the base sequence of the coding strand and the base sequence of the mRNA (ignoring the fact that mRNA will contain uracil instead of thymine) is

a) Complementary b) Identical c) anti-parallel d) none of the above

Ans: Complementary

12. The RNA strand synthesized during transcription elongates until

a) The entire chromosome has been copied into RNA

b) The RNA polymerase runs into the next gene

c) An intron encountered on the DNA template strand

d) A specific termination sequence is reached on the DNA template strand

Ans: A specific termination sequence is reached on the DNA template strand

13. Transcriptional regulation is controlled by

a) methylation of DNA b) number of genes in the genome c) poly A tail d) introns

Ans: methylation of DNA

14. ESTs are obtained through

a) Genomic DNA library b) cDNA library c) RT-PCR d) all the above

Ans: cDNA library

15. The only one start codon is ----------

(a) GAT (b) ATG (c) TAG (d) GTA

Ans: ATG

16. In prokaryotes, the matured mRNA is

a) Identifcal to the initial mRNA b) Shorter than the initial mRNA c) Longer than the initial mRNA d) None of the above

Ans: Identifcal to the initial mRNA

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Web Sites

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- Virtual Cell: http://www.life.uiuc.edu/plantbio/cell/
- www.kbiotech.com
- www.johnkyrk.com

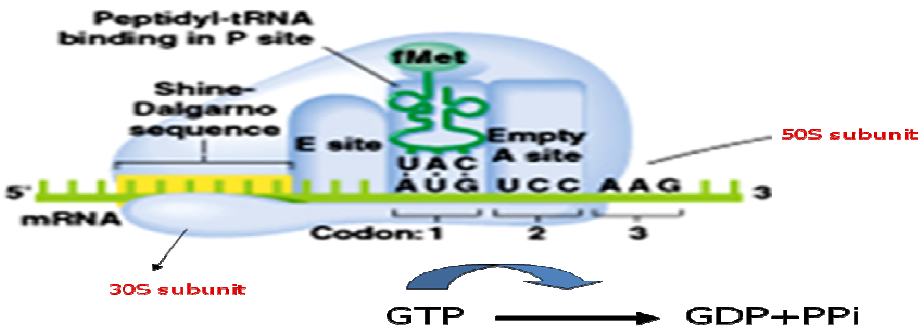
Lecture.25

Fatty acid and triacyl glycerol biosynthesis

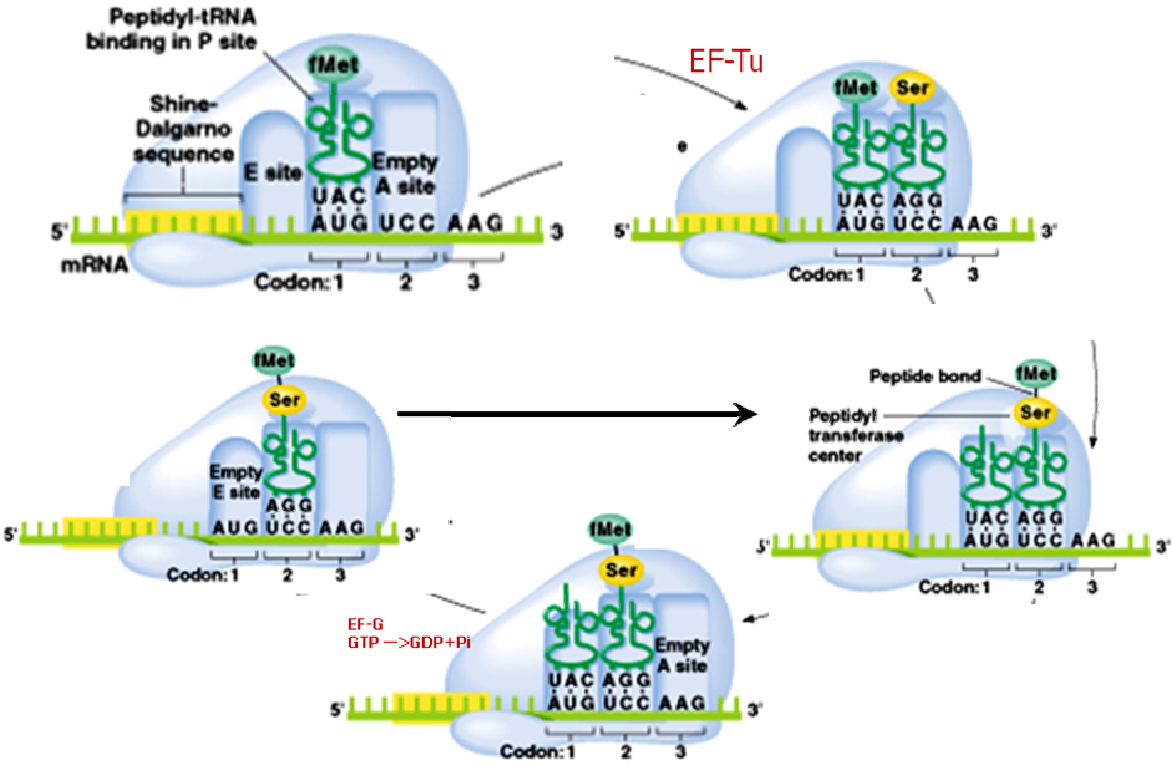
The synthesis of every protein molecule in a cell is directed by intracellular DNA. There are two aspects to understand how this is accomplished – Information aspect and Chemical aspect. Information aspect meant the mechanism by which a base sequence in a DNA molecule is translated into an amino acid sequence of a polypeptide chain. The chemical aspect refers to the actual process of synthesis of the protein: the means of initiating synthesis; linking together the amino acids in the correct order; terminating the chain; releasing the finished chain from the synthetic apparatus; folding the chain, and often postsynthetic modification of the newly synthesized chain. The overall process is called as Translation. Protein synthesis can be divided into three stages: 1. polypeptide chain initiation, 2. chain elongation and 3. Chain termination.

1. Initiation

In bacteria protein synthesis begins by the association of one 30S subunit (not the 70S ribosome), one mRNA molecule, a charged $tRNA^{fMet,}$, three proteins known as initiation factors and guanosine 5'-triphosphate (GTP). These molecules comprise the 30S preinitiation complex. Following formation of the 30S preinitiation complex, a 50S subunit joins to the 30S subunit to form a 70S initiation complex. This joining process requires hydrolysis of the GTP contained in the 30S preinitiation complex. There are two tRNA binding sites which overlap the 30S and 50S subunits. These sites are called as aminoacyl or A site and the peptide or P site; each site consists of a collection of segments of S and L proteins and 23S rRNA. The 50S subunit is positioned in the 70S initiation complex such that the $tRNA^{fMet,}$ which was previously bound to the 30S preinitiation complex, occupies the P site of the 50S subunit. Positioning $tRNA^{fMet,}$ in the P site fixes the position of the anticodon of $tRNA^{fMet,}$ such that it can pair with the initiator codon in the mRNA. Thus, the reading frame is unambiguously defined upon completion of the 70S initiation complex.



The A site of the 70S initiation complex is available to any tRNA molecule whose antiocodon can pair with the codon adjacent to the initiation codon. However, entry to the A site by the tRNA requires a helper protein called an elongation factor (EF), specifically EF-Tu. After occupation of the A site a peptide bond between fMet and the adjacent amino acid can be formed. Once it was thought that the blockage of NH_2 group of fMet by the formyl group was responsible for peptide bond formation between the COOH group of fMet and the NH_2 group of the adjacent amino acid. However, in eukaryotes the starting amino acid is Met and not fMet and protein synthesis proceeds in the correct direction. Presumably, the relative orientation of the two amino acids in the A and P sites determines the linkage that is made.



The peptide bond is formed by an enzyme complex called peptidyl transferase. The active site of peptidyl transferase consists of portions of several proteins of the 50S subunit. As the peptide bond is formed, fMet is cleaved from the $tRNA^{fMet,}$ in the P site. After the peptide bond forms, an unchanged tRNA occupies the P site and a dipeptidyl-tRNA is in the A site. At this point three movements, which together comprise the translation step, occur:

1. The deacylated $tRNA^{fMet,}$ leaves the P site.

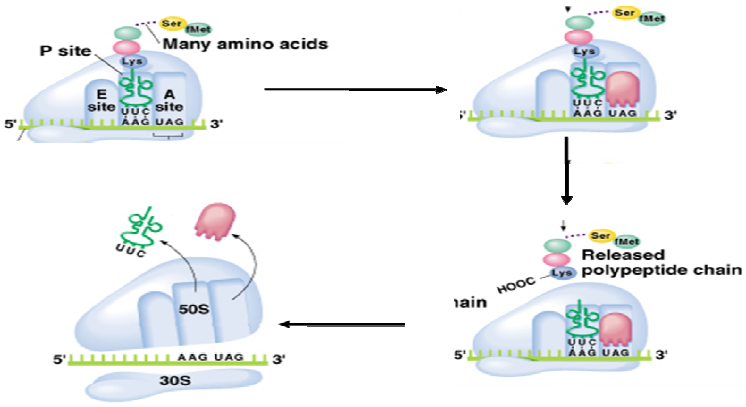
2. The peptidyl-tRNA moves from the A site to the P site and

3. The mRNA moves a distance of three bases in order to position the next codon at the A site. The translocation step requires the presence of another elongation protein EF-G and hydrolysis of GTP. The movement of the mRNA by three bases is probably dependant on the movement of tRNA from the A site to the P site and in fact, it is likely that mRNA translocation is a consequence of tRNA motion.

After translocation has occurred, the A site is again available to accept a charged tRNA molecule having a correct anticodon. If a $tRNA^{Met,}$ molecule, were to enter the A site (because an internal AUG site were present), protein synthesis would stop because a peptide bond cannot form with the blocked NH_2 group of fMet. However, in as much as the factor EF-Tu is needed to facilitate tRNA entry into the A site, this misadventure is prevented, since EF-Tu cannot bind to $tRNA^{fMet,}$.

When a chain termination codon is reached, there is no aminoacyl-tRNA that can fill the A site and chain elongation stops. However, the polypeptide chain is still attached to the tRNA occupying P site. Release of the protein is accomplished by release factors (RF), proteins that in part respond to chain termination codons. There are two such release factors in *E. coli* – RF1, which recognizes the UAA and UAG codons and RF2, which recognizes UAA and UGA. Why the number of release factor is not one (ie. useful to all 3 codons) or three (ie., one for each stop codon) is not known (in eukaryotes, there is only one release factor). Each release factor forms an activated complex with GTP; this complex binds to a termination codon and alters the specificity of peptidyl transferase. In the presence of release factors peptidyl transferase catalyses the reaction of the bound peptidyl moiety with water rather than with the free aminoacyl-tRNA. Thus the polypeptide chain, which has been held in the ribosome solely by the interaction with the

tRNA in the P site, is released from the ribosome. The 70S ribosome dissociates into 30S and 50S subunits and the system is ready to start synthesis of a second chain.



Differences between protein synthesis in eukaryotes and prokaryotes

1. In eukaryotes, the initiating amino acid is methionine (Met) and not fMet. The initiating tRNA, which responds only to AUG, is designated $tRNA^{Met}_{init}$, or $tRNA^{Met}_{f}$ to distinguish it from the $tRNA^{Met,}$ used in translating internal AUG codons.
2. At least nine initiation factors plus GTP are required for binding of $tRNA^{Met,}_{f}$, to the preinitiation complex. Two of these are similar to the factors in *E. coli* that prevent binding of the large subunit to the small subunit. One of these factors, eIF3, is a larger complex containing nine protein subunits and having a molecular weight of 7 X 10^5. An enzyme called initiating tRNA hydrolase, which cleaves the bond between methionine and $tRNA^{Met,}_{f}$ removes the initial tRNA molecule after the first peptide bond forms, is also present in the complex. In prokaryotes, the corresponding enzyme, tRNA deacylase, is a ribosomal component.
3. Binding of $tRNA^{Met,}_{f}$, must occur before mRNA can bind, whereas for prokaryotes the mRNA can bind either before or after binding of the initiator tRNA. For binding of mRNA two other initiation factors are needed and ATP must be cleaved to form ADP and P_i. The reason for the cleavage of ATP is unknown. Binding occurs initially at or near the 5' cap and is mediated by a cap binding factor (which is unnecessary for uncapped viral mRNA). The fact that the AUG codon nearest the 5'

terminus is almost always the initiating codon is a significant difference between prokaryotes and eukaryotes and plays important role in metabolic regulation.

4. More factors are needed for binding of the 60S subunit than for binding of the bacterial 50S subunit.
5. At least four elongation factors are needed by eukaryotes. These factors probably differ in structure and size in different tissues, often forming aggregates containing as many as 50 monomers.
6. Little is know about termination in eukaryotes, thoughs release factors have been purified. Surprisingly, release in *in vitro* systems requires the presence of one of four tetranucleotides – UAAA, UAGA, UGAA or UAGG.
7. The most striking difference is that transcription and translation is not couple in eukaryotes whereas it is coupled in prokaryotes. In eukaryotes, the mRNA is synthesized in nucleus and then transported to cytoplasm where the ribosome located.
8. In prokaryotes, degradation of mRNA occurs continuously and while translation is in process. The half-life of a typical bacterial mRNA is about 1.8 minutes. Eukaryotic mRNA is very stable- possibly because of the 5' cap. Degradation occurs very slowly and a typical half-life is several hours.

Questions

1. In eukaryotes, translation occurs in

a) Cytoplasm b) nucleus c) cellular compartment d) all the above

Ans: Cytoplasm

2. In eukaryotes, nascent mRNA is usually

a) polycistronic b) monocistronic c) dicistronic d) tricistronic

Ans: monocistronic

3. The templates used for protein synthesis is

a) genic DNA b) mRNA c) rRNA d) tRNA

Ans: mRNA

4. A sequence of translatable codons are referred as

a) ORF b) RBS c) promoter d) enhancer

Ans: ORF

5. The mRNA those are stable for hours or even days are synthesized in

a) Eggs b) muscles c) bones d) all the above

Ans: eggs

6. The role of modification of 5' end of mRNA with 7-methylguanosine cap is

a) Ribosome binding b) aligning P site c) aligning A site d) mRNA stability

Ans: ribosome binding

7. The maximum portion (60-65%) of total RNA consists of

a) mRNA b) tRNA c) rRNA d) hnRNA

Ans: rRNA

8. Which site of the ribosomes binds with incoming charged tRNAs during elongation?

a) A (aminoacyl) site b) P (peptidyl tRNA) site c) GTPase domain d) peptidyl transferase domain

Ans: A (aminoacyl) site

9. In eukaryotes, the small subunit of ribosome along with cap binding proteins (CBP) (eIF-4F) binds at

a) Shine-Dalgarno sequence b) Kozak sequence (ACCAUGG) c) promoter

d) Enhancer

Ans: Kozak sequence (ACCAUGG)

10. Examples of programmed misreading, where the normal interpretation of the sequence of codons is suppressed is

a) Read through b) selenocystein insertion (at termination codon- UGA; called as selenocystein insertion sequence) c) frame shifting d) all the above

Ans: all the above

11. A protein composed of 300 amino acids would be encoded by an mRNA of

a) 300 nucleotides b) 400 nucleotides c) 900 nucleotides d) 400 nucleotides

Ans: 900 nucleotides

12. The three types of RNA responsible for forming part of the structure upon which protein synthesis occurs, dictating the order of amino acids in the growing protein chain and transporting amino acids to their site of protein synthesis, respectively are

a) mRNA, rRNA, tRNA b) tRNA, mRNA, rRNA

c) rRNA , mRNA, tRNA d) rRNA, tRNA, mRNA

Ans: rRNA, mRNA, tRNA

13. Anticodon is a part of the tRNA molecule that is responsible for specific attachment to the

a) mRNA b) Amino acid c) Ribosomal complex d) rRNA

Ans: mRNA

14. Which of the following sentence is incorrect

a) Eukaryotic nuclear mRNAs are monocistronic

b) mRNA that carry the information for more than one type of protein is not found eukaryotic cells

c) A bacterial mRNA can carry the information for more than one type of protein

d) All the above

Ans: mRNA that carry the information for more than one type of protein is not found eukaryotic cells

15. In prokaryotes, many ribosomes translate an mRNA simultaneously, forming a structure called

a) polysome b) polycistron c) 30S initiation complex d) all the above

Ans: polysome

16. In Eukaryotes, to locate the initiator codon, the small ribosomal subunit has to first locate

a) The 5'-methyl guanosine cap b) Ribosome binding site c) Shine-Dalgarno sequence d) all the above

Ans: Shine-Dalgarno sequence

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Lecture.26

History of Biotechnology

The word "biotechnology" was first coined in 1917 by a Hungarian agricultural engineer, Karl Ereky. He used it to describe a system for raising pigs on sugar beets as their primary food source. He defined Biotechnology as "all lines of work by which products are produced from raw materials with the aid of living things". He defined such a new terminology since most of the industries use mechanical devices - machines - to make things, biotechnology uses living organisms to make products of economic value.

However, it seems from this definition that Biotechnology is ancient or even prehistoric, going back at least 12,000 years. The earliest evidence for domestication of animals is the dog, ca. 12,000 years ago. The above said definition can also be used for most agricultural production. The earliest evidence for the invention of agriculture is about 11,000 years ago in SW Asia (the Fertile Crescent), North China, and South China.

Apart from the traditional cultivation of crops and raising of livestock, there are a number of other older technologies that can also be characterized as biotechnology:

1. **Fermentation**: Another prehistoric or ancient biotechnology is the use of yeast. All of the food processing systems such as brewing beer, wine-making, baking bread rely on yeast to alter the properties of a raw material.
2. **Sewage treatment**: Organic wastes are degraded by the action of a complex community of microbes.

But the concept of this biotechnology have been altered beyond this original vision by a series of developments in the life sciences. These developments center on the ability to:

- isolate genes from any organism
- modify and manipulate these genes
- put these altered genes back into various organisms

This is the heart of the "biological revolution" that is going on at the moment.

The 19th century and early 20th century saw the development of many mechanical devices, and the 20th century had the era of chemicals and electronics. But in the 21st century we will likely see our understanding of biological processes put to greater use in changing how we live our lives. This led to the development of different branches of biotechnology in plant science such as

- ✓ plant tissue culture
- ✓ genetic engineering or recombinant DNA technology
- ✓ DNA marker technology or Marker assisted breeding or molecular breeding
- ✓ Bioinformatics
- ✓ Environmental biotechnology
- ✓ Biofertilizers and biopesticides
- ✓ Industrial biotechnology
- ✓ Microbial biotechnology
- ✓ Food biotechnology and fermentation technology
- ✓ genetical genomics and systems biology
- ✓ biosafety, bioethics, intellectual property rights (IPR) with respect to biotechnology etc.,

As that of plant biotechnology, biotechnology has various fields or branches in medicine, pharmaceuticals and other branches of life sciences.

Definition of Biotechnology

Biotechnology has different meanings to different people. Some define biotechnology as the use and exploitation of microorganisms to produce products of industrial or commercial interest like beer brewing, cheese making, and production of sour milk. But is this the right definition for biotechnology? The answer is no, this is a narrow definition that can't describe biotechnology. the exploitation of genetically engineered microorganisms, centered on the technology of genetic engineering or recombinant DNA, constants modern biotechnology. So, in the light of the previous sentence, biotechnology is defined as the application of scientific and engineering principles to the processing of materials by biological agents to provide goods and services**.**

Many different scientific fields are responsible for emergence of this biological revolution. They are biochemistry, molecular biology, microbiology, physiology, computer science and what not?

Hence, a newer, refined, definition of biotechnology was offered by U.S. Office of Technology Assessment: "Any technique that uses living organisms to make or modify products, to improve plants or animals, or to develop microorganisms for specific purposes."

The United Nations Convention on Biological Diversity defines biotechnology as: "Any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use."

The ultimate goal of this course is to understand how those products of biotechnology are made and the scientific principles that underlie this revolution. This will also help you to assess the safety and merits of the various applications of this new technology. As with all new developments, there are many, some may think that this technology is dangerous. For some it is unethical or immoral to tamper with the genetic material, they believe this will lead to a loss of our own "genetic privacy", and that no one has the right to own and patent either organisms or genes. Others question the environmental consequences of these developments, that it will lead to the development of more potent pests, create new health problems because of allergens and toxins, and reduce biodiversity. Yet others question the socioeconomic consequences of biotechnology, for example the promotion of "corporate" farming, and the effects on less developed countries. We will examine these issues as we learn about the development of agricultural biotechnology.

Box 1. Selected historical events that lead to the development of biotechnology:

*(events in **bold** letters are must to remember)*

1810: Brown described nucleus as a central feature in plant cells.

1838: Schleiden and Schwann proposed cell theory.

1865: Mendal published his experimental results on the inheritance of traits in peas.

1869: Meischer reported that DNA is a major component of the nucleus.

1900s: Mendel's results are "rediscovered" and the science of genetics begins. Laws of heredity are confirmed in plants.

1875: Strasburger described chromosomes 1902: Sutton described chromosome theory

1902: Haberlandt first attempted plant tissue culture

1903: Buchner discovered the enzyme

1911: Morgan proposed the gene theory: genes are linearly arranged along chromosomes in a definite order.

1917: Karl Ereky coined the term biotechnology

1922: Knudson showed *in vitro* asymbiotic germination of orchid seeds

1928: Griffith discovered genetic transduction in bacteria.

1934: White demonstrated successful culturing of tomato roots

1935: Stanley isolated tobacco mosaic virus in crystalline form.

1941: Van Overbeek used first time the coconut milk which contains a cell division factor

1944: Avery, Mcleod and McCarty showed the significance of DNA in hereditary transmission in bacteria.

1944: Barbara McClintock discovered the transposable elements.

1952: Morel and Martine used meristem tip culture for production of virus free plants

1952: Morel and Martine shown first application of micrografting

1952: Chase and Hershey showed that the gene was DNA.

1953: Watson and Crick invented DNA double helix model.

1954: Sanger gave the complete structure of a protein molecule (insulin).

1957: Skoog and Miller discovered the regulation of organ formation by changing the ratio of auxin : cytokinin

1958: Beadle and Tatum discovered that one gene one polypeptide hypothesis- one gene regulates one definite chemical process.

1958: Crick proposed the central dogma of life or molecular biology

1959: Kornberg synthesized *in vitro* polydeoxiribonucleotides (DNA).

1960: Kanta showed first successful test tube fertilization in *Papaver rhoeas*

1960: Cocking developed enzymatic degradation of cell walls to obtain large number of protoplasts

1961: Crick produced direct evidence that the genetic code is a triplet one.

1961: Jacob and Monad discovered regulatory genes- operon concept.

1962: Murashige and Skoog has developed MS medium

1964: Guha and Maheswari developed first haploid plants from pollen grains of *Datura*

1968: Nirenberg and Khorana proposed genetic code for amino acids.

1968: Meselson and Yuan coined restriction endonuclease: a class of enzymes involved in DNA cleavage.

1970: Temin and Baltimore discovered reverse transcriptase.

1970: Power et al., first achieved the protoplast fusion

1970: Smith discovered first restriction endonulease from *Haemophillus influenzae* Rd. it was later purified and named as *HindII.*

1971: Nathans prepared first restriction map using *HindII* to cut circular DNA of SV40 into 11 specific fragments.

1972: Khorana et al., synthesized entire tRNA gene.

1972: Berg et al., produced first recombinant DNA molecule

1973: Boyer and Cohen established recombinant DNA technology.

1975: O'Farrel developed high resolution two-dimensional gel electrophoresis procedure, which led to the development of proteomics.

1978: Nathans, Smith and Arber discovered restriction enzymes and awarded Nobel prize.

1980: Eli Lilly Co., has commercially produced human insulin through genetic engineering in bacterial cells.

1981: Larkin and Scowcroft introduced somaclonal variation.

1983: Engineered Ti plasmids used to transform plants.

1983: Kary Mullis discovered polymerase chain reaction (PCR).

1984: Jeffreys introduced DNA fingerprinting to identify individuals.

1984: De Block et al., and Horsch et al., shown transformation of tobacco with *Agrobacterium* and transgenic plants were developed.

1986: Powell-Abel et al., shown TMV resistant tobacco and tomato transgenic plants developed using cDNA of coat protein gene of TMV.

1987: Development of biolistic gene transfer method; isolation of Bt gene from *Bacillus thrungiensis.*

1990: Human Genome Project officially initiated and physical and genetic map was published in 2000.

1995: The first genetically engineered potato, resistant to the Colorado potato beetle is sold in Canada. Canada is the first country in the world to grow biotech crops.

1997: Nuclear cloning of a mammal, a sheep – dolly, with a differentiated cell nucleus was done. Blattner et al., completely sequenced *E. coli.*

1998: Genome sequencing of *Caenorhabditis elegans* completed.

2000: Craig Venter and Francis Collins announced the first draft human genome sequence.

2002: Complete draft genomic sequence of rice published.

2007-2009: Initiatives on genome sequencing in many agriculturally important crops such as cotton, tomato etc.,

(This is just to list a few well known historical events. The list is still lengthy and growing)

Box 3. Flavr savr tomato.

The tomato has been one of the first targets of plant biotechnology for a number of reasons: 1. Tomatoes are in the same family, the Solanaceae, as tobacco. Tobacco was widely used as a model for plant transformation, so it was fairly easy to develop transformation systems for tomato. 2. There are a number of characteristics of fresh market tomatoes that consumers are unhappy about: quality, flavor and shelf life. Most of the year, fresh tomatoes bought in the store are of very poor quality and have little resemblance to fresh garden tomatoes. The basis for the FLAVR SAVR tomato, the first whole food product of biotechnology, was reducing the expression of polygalacturonase (PG) in tomato fruit and thus slowed down the softening of the fruit. They used the antisense RNA approach to produce plants with reduced expression of PG in fruit. After several reviews by regulatory agencies (like FDA), this product was launched in late 1994.

Apart from these two examples, there are several other biotech products that are commercially available such as Bt cotton, Bt corn, herbicide resistant crops, tissue cultured banana, orchids and number of other horticultural and forestry crops, VAM, P-solubilizing bacteria etc., Advance learners are requested to refer the references/websites given below to get more on these biotech products.

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Lecture.27

Fundamentals of Micropropagation

Fundamentals of Micropropagation

Multiplication of genetically identical copies of a cultivar by asexual reproduction is called *clonal propagation*. In nature, clonal propagation occurs by apomixis (seed development without meiosis and fertilization) and/or vegetative propagation (regeneration of new plants from vegetative parts). Tissue culture has become popular method for vegetative propagation of plants. Aseptic method of clonal propagation is called as *Micropropagation* and it offer the advantage of large number of true-to-type plantlets can be produced with relatively short time and space from a single individual. Since any small part of the plant can be used to produce complete plantlets, this method is called as micropropagation. It is the fact that micropropagation is the one of the commercially viable methods of clonal propagation of the horticultural crops. E.g. Orchids.

Cellular Totipotency

The inherent potentiality of a plant cell to give rise to a whole plant is described as cellular totipotency. This is a capacity which is retained even after a cell has undergone final differentiation in the plant body. In plants, even highly mature and differentiated cells retain the ability to regenerate to a meristematic state as long as they have an intact membrane system and a viable nucleus. This is contradicting to animals, where differentiation is generally irreversible.

For a differentiated cell, to express its totipotency, it first undergoes dedifferentiation followed by redifferentiation. The phenomenon of a mature cell reverting to the meristematic state and forming undifferentiated callus tissue is termed ‘dedifferentiation’. The phenomenon of conversion of component cells of callus tissue to whole plant or plant organs is called as ‘redifferentiation’.

Explant and explant selection

A plant organ or piece of tissue used to initiate a culture. Almost all parts of plant are amenable to *in vitro* plant regeneration (E.g. Tobacco). However, in certain plants some organs may be more regenerative than the others (e. g. in *Glycine max*, the hypocotyls exhibits higher potentiality for shoot formation than the root segments).

The regenerability of an explant is influenced by several factors:

1. Organ from which it is derived.
2. The physiological state of explant
3. Size of the explant
4. Orientation of the explant on the medium and
5. Its inoculation density.

Sterilization and its techniques

Sterilization is the process of inactivating or removing all living organisms from a substance or surface. Different kinds of sterilization procedures were adapted in plant tissue culture. They are,

1. **Heating**

Dry heat (e.g. Hot air oven) is used for glassware and **Steam or moist or wet heat (e.g. Autoclave)** is used for sterilization of media and fermenter vessels.

2. **Radiation**

UV radiation and high efficiency particulate air (HEPA) filter in laminar air-flow chamber used for sterilization of transfer area.

3. **Chemicals**

95 % ethanol used for sterilizing forceps, scalpels, needles etc., Hypochlorite solutions (sodium or calcium) and antibiotics are used as sterilizing agents to disinfect plant tissues.

4. **Ultra filtration**

The solution of thermolabile compounds (e.g. vitamins, antibiotics etc) is sterilized by membrane filtration.

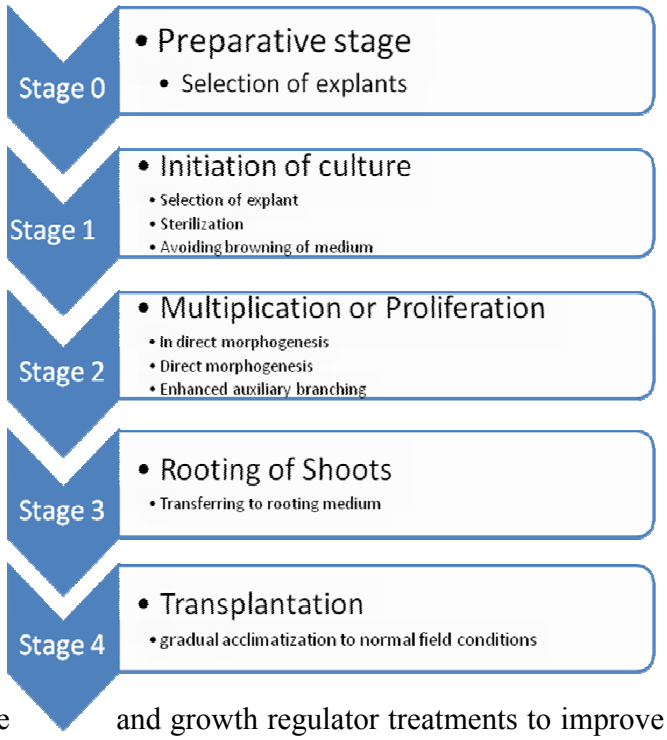
Explants used in Micropropagation

Different kinds of explants were used in micropropagation. For example, in case of orchids, shoot tip (*Anacamptis pyramidalis, Aranthera, Calanthe, Dendrobium),* axillary bud (*Aranda, Brassocattleya, Cattleya, Laelia)*, inflorescence segment (*Aranda, Ascofinetia, Neostylis, Vascostylis)*, lateral bud (*Cattleya, Rhynocostylis gigantean)*, leaf base (*Cattleya)*, leaf tip (*Cattleya, Epidendrum),* shoot tip (*Cymbidium, Dendrobium, Odontioda, Odontonia),* nodal segment (*Dendrobium),* flower stalk segment (*Dendrobium, Phalaenopsis)* and root tips (*Neottia, Vanilla)* are being used in micropropagation.

Stages in micropropagation

Micropropagation generally involves five stages. Each stage has its own requirements.

Stage 0: Preparative stage



This stage involves the preparation of mother plants to provide quality explants for better establishment of aseptic cultures in stage 1. To reduce the contamination problem in the subsequent stages, mother plant should be grown in a glasshouse and watered so as to avoid overhead irrigation. This will also reduce the need for a harsh sterilization treatment. Stage 0 also includes exposing the stock plants to suitable light, temperature and growth regulator treatments to improve the quality of explants. In the case of photosensitive plants it may be possible to obtain suitable explants throughout the year by controlling photoperiod in the glasshouse. For

example, red-light treated plants of *Petunia* provided leaf explants which produced up to three times as many shoots as did the explants from untreated plants. Further, selection of mother plants which contains the trait of interest should also be ensured.

Stage 1. Initiation of culture

1. **Explant:** The nature of explant to be used for *in vitro* propagation is governed by the method of shoot multiplication. For enhanced axillary branching, only the explants which carry a pre-formed vegetative bud are suitable. When the objective is to produce virus-free plants from an infected individual it becomes necessary to start with sub-millimeter shoot tips. If the stock is virus-tested or virus eradication is not necessary, then the most suitable explant is nodal cuttings. Small shoot-tip explants have a low survival rate and show slow initial growth. Meristem tip culture may also result in the loss of certain horticultural characteristics which are controlled by the presence of virus, such as the clear-vein character of the Geranium cv. Crocodile. Generally, the clear vein character is transmitted in petiole-segment culture but not in shoot-tip culture.
2. **Sterilization:** This operation needs to be done under sterilized conditions ie., inside Laminar airflow chamber. Special precautions need to be taken when explants are derived from field-grown materials, which is often necessary in cloning an elite tree. In such cases an ideal approach would be to take cuttings from the selected plant and grow them in greenhouse. It is necessary to treat the explants with suitable sterilizing agents such as 0.1% $HgCl_2$ or sodium hypochlorite or other sterilants for 1- 10 minutes (the duration may vary with respect to the species, which needs to be standardized for each species). Then wash with double distilled sterilized water with five or six times Discarding the surface tissues from plant materials while preparing the explants also minimizes the loss of cultures due to microbial contamination. Appropriate size of the explants may be prepared under aseptic conditions and then it will be inoculated into the suitable medium.
3. **Browning of medium:** A serious problem with the culture of some plant species is the exudation of phenolic compounds leached out from the cut surface of the explant. It turns the medium dark brown and is often toxic to the tissues. This problem is

common with the adult tissues from woody species. This problem can be circumvented by adding activated charcoal, ascorbic acid, PVPP etc., into the medium.

3. Stage 2. Multiplication / Proliferation

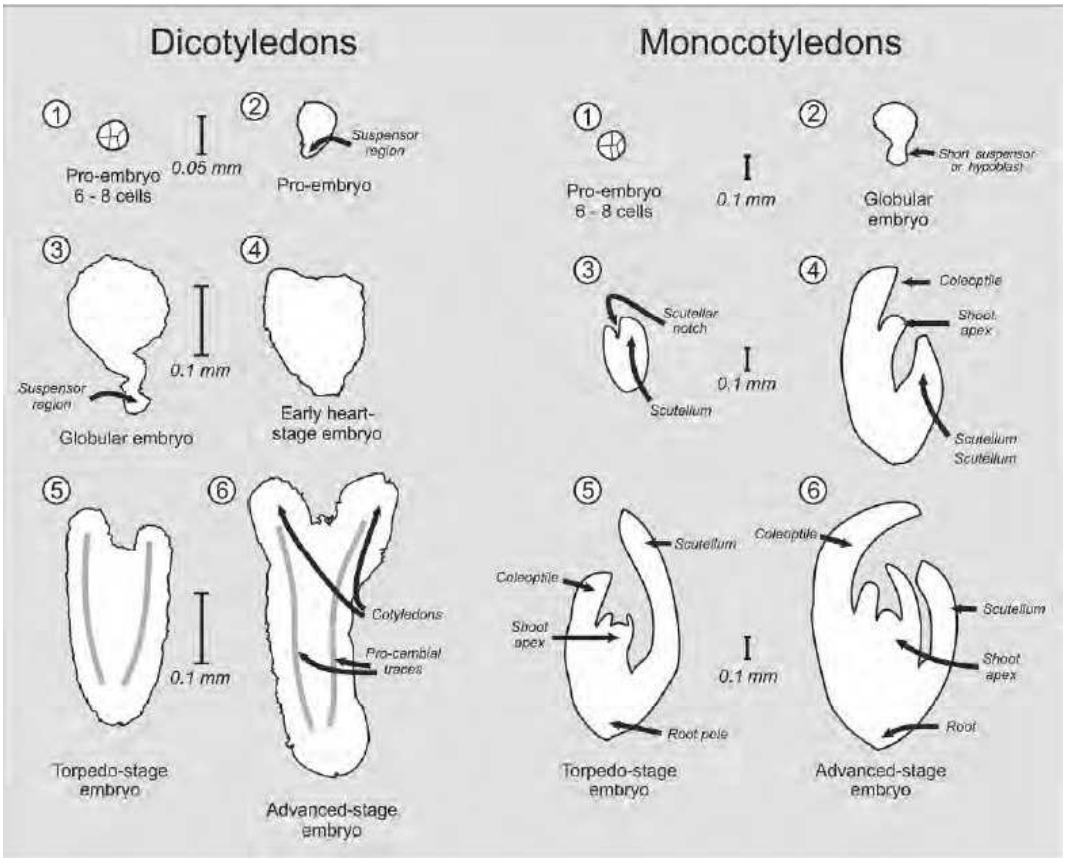
This is the most crucial stage since it is the point at which most of failures in micropropagation occur. Broadly three approaches have been followed to achieve *in vitro* multiplication.

1. Through callusing (in direct morphogenesis): The potentiality of plant cells to multiply indefinitely in cultures and their totipotent nature permit a very rapid multiplication of several plant types. Differentiation of plants from cultured cells may occur via shoot-root formation (organogenesis) or somatic embryogenesis. Somatic embryogenesis is most appealing from a commercial angle. A somatic embryogenesis system once established lends itself to better control than organogenesis. Since somatic embryos are bipolar structures, with defined root and shoot meristems, the rooting stage required for microshoots gets eliminated. Above all, somatic embryos being small, uniform and bipolar are more amenable to automation at the multiplication stage and for field planting as synthetic seeds, offering cost advantages from labour savings, can also be stored through cold storage, cryopreservation or dessication for prolonged periods. These characteristics make somatic embryogenesis potentially a less expensive and flexible system for micropropagation. The most serious objection against the use of callus cultures for shoot multiplication is the genetic instability of their cells since it may lead to somaclonal variation.

2. Adventitious bud formation (direct morphogenesis): Buds arising from any place other than leaf axil or the shoot apex are termed adventitious buds. The shoots differentiated from calli should also be treated as adventitious buds. In many crops, vegetative propagation through adventitious bud formation from root (blackberry, raspberry) and leaf (*Begonia, Crassula)* cuttings is standard horticultural practice. In such cases the rate of adventitious bud development can be considerably enhanced under culture conditions. For most bulbous plants (e.g. Lilley) adventitious bud

formation is the most important mode of multiplication and the best explants are obtained from bulb scales. A serious problem may arise when this method of propagation is applied to varieties which are genetic chimeras. Adventitious bud formation involves the risk of splitting the chimeras leading to pure type plants. For example, in variegated geranium cv. Mme Salleron, the chimera is perpetuated in meristem culture but broken down in petiole culture.

3. Enhanced axillary branching: In cultures the rate of shoot multiplication by enhanced axillary branching can be substantially enhanced by growing shoots in a medium containing a suitable cytokinin at an appropriate concentration with or without auxin. Due to continuous availability of cytokinin, the shoots formed by the bud, present on



Stages of somatic embryogeneis in dicotyledons and monocotyledons

4. The explant, develops axillary buds which may grow directly into shoots. This process may be repeated several times and the initial explant transformed into a mass of branches. This method is the most preferred one for clonal propagation of the mother plant.

4. Stage 3. Rooting of shoots

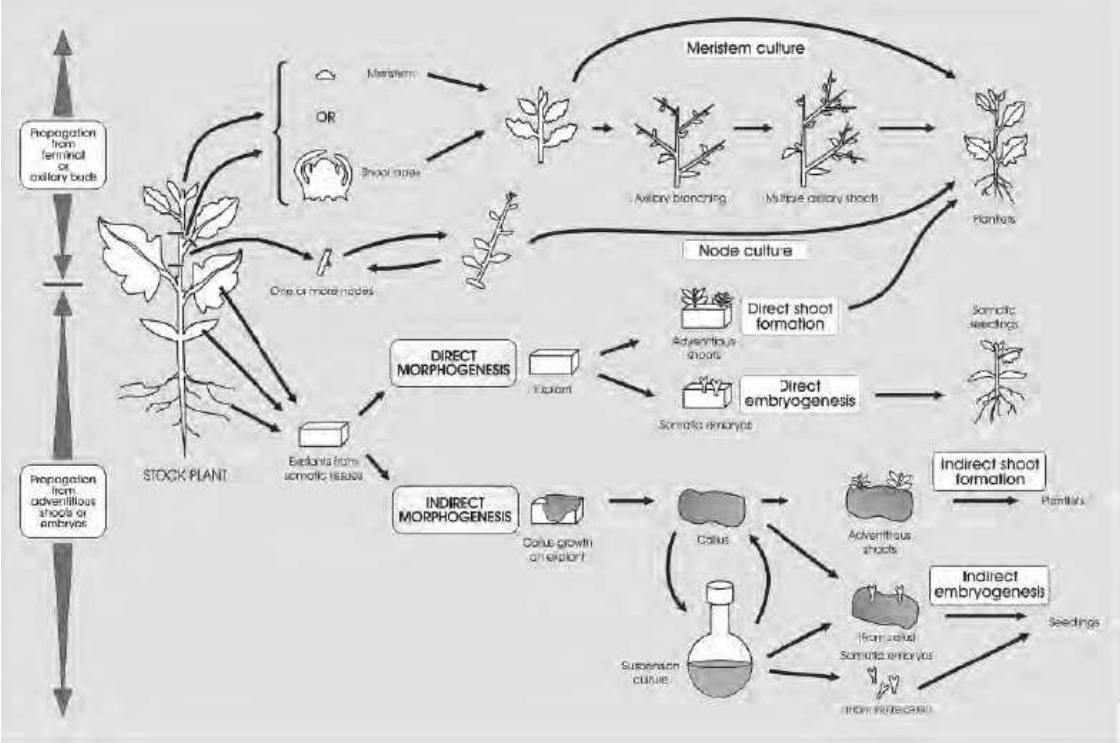
Somatic embryos carry a pre-formed radicle and may develop directly into plantlet. However, these embryos often show very poor conversion into plantlets. They require an additional step of maturation to acquire the capability for normal germination. Adventitious and axillary shoots developed in cultures in the presence of a cytokinin generally lack roots. To obtain full plants the shoots must be transferred to a rooting medium which is different from the shoot multiplication medium, particularly in its hormonal and salt compositions. For rooting, individual shoots measuring 2 cm in length are excised and transferred to the rooting medium.

5. Stage 4. Transplantation

The ultimate success of commercial propagation depends on the ability to transfer plants out of culture on a large scale, at low cost and with high survival rates. The plants multiplied *in vitro* are exposed to a unique set of growth conditions (high levels of inorganic and organic nutrients, growth regulators, sucrose as carbon source, high humidity, low light, poor gaseous exchange) which may support rapid growth and multiplication but also induce structural and physiological abnormalities in the plants, rendering them unfit for survival under *in vivo* conditions. The two main deficiencies of *in vitro* grown plants are – poor control of water loss and heterotrophic mode of nutrition. Therefore, gradual acclimatization is necessary for these plants to survive transition from culture to the greenhouse or field. During acclimatization the *in vitro* formed leaves do not recover but the plant develops normal leaves and functional roots. While transferring out shoots/roots their lower part is gently washed to remove the medium sticking to them. The individual shoots or plantlets are then transferred to potting mix and irrigated with low concentration of inorganic nutrients. This probably recommissions the photosynthetic machinery of plants, enabling them to withstand the subsequent reduction in the ambient relative humidity and survive under field conditions. A variety of potting mixtures such as peat, perlite, polystyrene beads, vermiculate, fine bark, coarse sand etc. or their

mixtures in different combinations are used for transplantation. For initial 10-15 days, it is essential to maintain high humidity (90-100%) around the plants, to which they got adapted during culture. The humidity is gradually reduced to ambient level over a period of 2-4 weeks.

Schematic representation of micropropagation



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Lecture.28

Micropropagation: Applications and Scope for commercialization;Importance of Biotechnology in Horticulture

Advantages of micro propagation with respect to commercialization

1. Clonal mass propagation - extremely large numbers of plants can be produced. Rather than getting 10000 plants per year from an initial cutting in vegetative propagation, one can obtain more than 1,000,000 plants per year from one initial explant through micropropagation. Hence, there is a huge scope for commercialization of most demanded horticultural crops.

2. **Culture is initialized from small parts of plants** – so no need of much space: from 1 m^2 space in culture room, 20000 - 100000 plants can be produced per year.

3. **Production of disease and virus free plantlets-**This leads to simplification of international exchange of plants

4. Micropropagation enables growers to increase the production of plants that normally propagate very slowly such as *Narcissus* and other bulbous crops.

5. Introduction of disease free new cultivars is possible through micropropagation

6. Vegetative propagation of sterile hybrids can be used as parent plants for seed production. Eg. Cabbage

7. One of the rapid methods for cloning of disease free trees.

8. *In vitro* cultures can be stored for long time through cryopreservation.

9. Breeding cycle can be shortened. E.g. production of doubled haploid

10. Germplasm storage

11. Micropropagation may help in crop improvement program: e.g. selection for salt or drought tolerance using somaclonal variation.

12. CMS lines can be maintained using micropropagation

13. Micropropagation will be useful in production of somatic hybrids

14. Micropropagation can also be used to produce artificial seeds

15. Micropropagation will be employed to develop hybrids where incompatibility is a problem using embryo rescue.

16. *In vitro* grafting is one of the micropropagation techniques which is useful to get rid of virus or rooting problems.
17. Micropropagation can be used to generate *in vitro* mutants.

Disadvantages of micropropagation

1. Expensive laboratory equipment and service
2. No possibility of using mechanization
3. Plants are not autotrophic
4. Poor Acclimatization to the field is a common problem (hyperhydricity)
5. Risk of genetic changes if 'de novo' regeneration is used
6. Mass propagation cannot be done with all crops to date. In cereals much less success is achieved.
7. Regeneration is often not possible, especially with adult woody plant material. More problems in inducing rooting
8. Epigenetic modifications in the culture

Benefits of Biotechnology in horticulture

1. Several numbers of horticultural crops have been commercialized from biotechnological endeavor. E.g.
    a. Papaya genetically engineered fro papaya ringspot virus. This product has revived the Hawaiian papaya industry, which was devastated by the virus in the 1990's.
    b. Calgene, USA has released Flavr Savr tomato which has gene silencing of polygalacturonase, an enzyme implicated in fruit ripening.
2. Genetic engineering of sweet corn and potato with bt genes (genes isolated from *Bacillus thruingiensis*) for resistance against colorado potato beetle and corn ear worm.
3. Biotechnology is contributing in the development of sensitive diagnostic techniques (such as ELISA, PCR etc.,) for the identification of plant pathogens.
4. Good progress has been made in developing resistance to codling moth and fireblight in apple, plum pox virus in plum, crown gall and codling moth in

walnut, citrus tristeza virus (CTS) in citrus and Pierce's disease in grapevine using transformation technology.

5. Similarly, projects to increase the content of vitamins, minerals or nutraceuticals in horticultural products are in progress.
6. Several ornamental plants, including carnation, rose and gerbera have been engineered for modified flower colour.
7. Research on genes controlling the different biochemical pathways for various floral fragrances in snapdrogan, petunia and rose.
8. Engineering of plants to delay leaf senescence (yellowing) is also being pursued in ornamental crops by repressing cytokinins, which is involved in loss of chlorophyll.
9. Secondary metabolite production by *in vitro* culture is being done in many plants. E.g. shikonin production.

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Lecture.29

Meristem tip culture

Meristem tip culture

Most of the crop plants, especially those propagated by vegetative means, are systematically infected with one or more pathogens. A large number of viruses are not transmitted through seeds. In such cases, it should be possible to obtain virus free plants from infected individuals by using their seeds as the propagules. However, most of the horticultural plants are propagated through vegetative means. Hence, there is a need to develop a method of virus free plant in those plants.

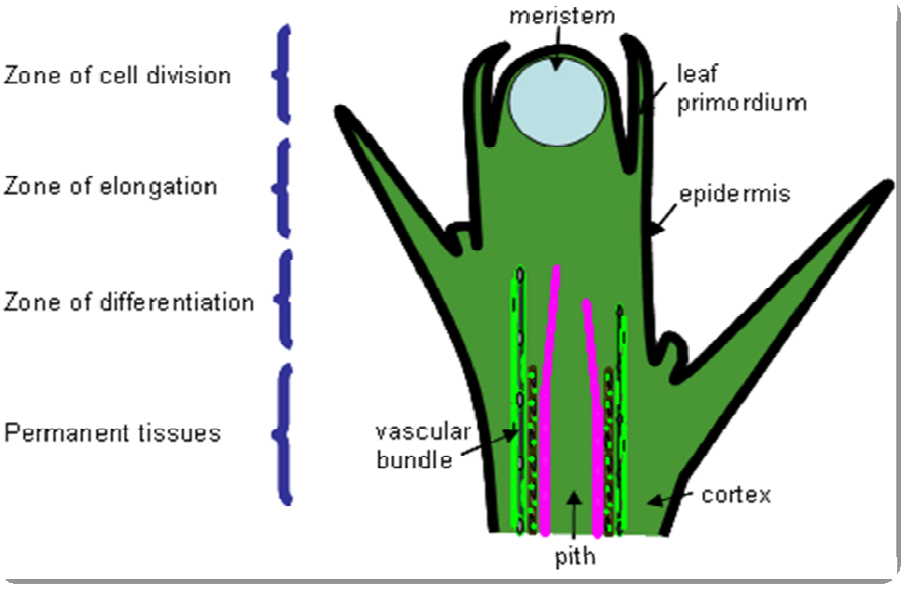
It is well known that the distribution of viruses in plants is uneven. In infected plants the apical meristems are generally either free or carry a very low concentration of the viruses. The reasons proposed for the escape of the meristem from virus invasion are

a. Viruses readily move in plant body through the vascular system which is absent in meristem. The alternate method of cell-to-cell movement of the virus through plasmodesmata is rather too slow to keep pace with the actively growing tip.
b. High metabolic activity in the actively dividing meristem cells does not allow virus replication.
c. The virus inactivating systems in the plant body has higher activity in the meristem than in any other region.
d. High endogenous auxin level in the shoot apices may inhibit virus multiplication.

The knowledge of the gradient virus distribution in the shoot tips enabled Holemes (1948) to obtain virus free plant from infected individuals of *Dahlia* through shoot-tip culture. Although mainly used for virus elimination, meristem tip culture has also enabled plants to be freed from other pathogens including mycoplasmas, bacteria and fungi (and certain viroids). Before the meristem tip culture technique was developed the in vivo eradication of viruses was achieved by heat treatment of whole plants. In a majority of cases heat therapy is combined with meristem tip culture in order to produce the greatest number of plants that are “virus free”.

Only the meristematic dome and 1 pair of subtending leaves should be excised. If larger pieces are taken, it is likely that the virus will be transmitted. The size of a

meristem plus the subtending leaves ranges from 0.1-0.5 mm. The apical dome itself measures from 0.1-0.25 mm depending on the species. There is a balance in size. The



meristem tip must be small enough to eradicate viruses and other pathogens, yet large enough to develop into a shoot. Although roots may form on the shoot directly in the same medium, often the shoot has to be transferred to another medium in order for roots to develop. The term *meriste*m, *shoot ti*p, *meristem tip* are often interchanged. Here we will use the term *shoot tip* to refer to an apical tip ranging from 1-3 cm. The *meristem* is strictly the meristematic dome without any primordial leaves. The term *meristem tip* will be used to denote the meristem together with 1-2 primordial leaves and measuring between 0.1 and 0.5 cm in height. For plant treatment proper meristematic apex without adjacent leaf primordia (size - 0.2-1.0 mm) is used as starting explant (Figure).

Heat therapy

In plant tissue cultures viruses can also be eliminated with use of higher temperatures (heat treatment.).In such case explants are exposed to the incidence of higher temperatures, which are not lethal for plant cells, but they are lethal for viruses. Temperature range most commonly used in this therapy is 50-52 ^{o}C with exposition about 10-30 minutes. In case this method is applied on whole plants, lower temperatures have to be used (32-40 ^{o}C) with exposition about 4 - 30 days (depends on plant species and

virus type). Mostly combination of thermo-therapy and meristem culture is used for virus-free plants production (e.g., cassava, bananas, citruses, strawberry, Irish potato, apples, chrysanthemas, garlic). Heat therapy combined with meristem tip culture is able to eradicate viruses, bacteria, and fungi but does not remove viroids. Unlike viruses, viroids are RNA without a protein coat – thus they are known as 'naked' RNA and are very difficult to eradicate. Usually the infected plant must be destroyed.

Chemotherapy

Virus-free plants can be also obtained when antivirus compounds are added into nutrient solution (Ribavirin or 2-thiouracil).

Shoot tip grafting (STG) /Micrografting

In a number of species including those of citrus peach and apple attempts at meristem culture remained unsuccessful in virus elimination. As an alternative, shoot tips of 0.14-0.18 mm in length isolated aseptically from a diseased plant were grafted on to young etiolated root stock seedlings grown in vitro.

Virus Indexing

The biological assays are reasonably accurate, but too slow and difficult. Alternatively, techniques involving electron microscope, use of indicator plants, serology or a combination of both were developed for virus detection in plant tissues. Among serological techniques, enzyme linked immunosorbent assay (ELISA) and nucleic acid hybridization techniques are popular.

Examples for Plant species for which virus free plants produced through meristem tip culture

Plant species	Virus eliminated
Allium sativum	Garlic Mosaic Virus
Brassica oleracea	Cauliflower Mosaic Virus
Dahlia spp.	Complex viruses
Glycine max	Soybean mosaic virus
Musa spp.	Cucumber mosaic virus
Nicotiana tobaccum	Tobacco mosaic virus

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Lecture.30
Anther, Pollen and Embryo cultures

Anther Culture

The impact of haploid production in genetics and plant breeding has long been realized. Homozygous lines of the cross pollinating species and hybrids are highly desirable to increase the efficiency of selection and production of homozygous plants. The conventional method to produce homozygous plants is lengthy and laborious, requiring 7-8 recurrent cycles of inbreeding. Moreover, this approach is impractical for self-incompatible and male sterile and tree species. On the other hand, homozygous plants can be obtained in a single generation by diploidization of the haploid. This kind of production of stable, homozygous dihaploids or doubled haploids (DH) in a single generation equivalent to the F_{α} generation of pedigree breeding and thus considerably shortens the breeding cycle. However, their exploitation remained restricted because of the extremely low frequency with which they occur in nature. Spontaneous production of haploids usually occurs through the process of parthenogenesis (embryo development from unfertilized egg). Rarely, they reproduce male parent alone. This suggest that their origin through 'ovule androgenesis' (embryo development inside the ovule by the activity of the male nucleus alone). In vivo occurrence of androgenic haploids has been reported in *Antirrhinum, Nicotiana* etc. the artificial production of haploids was attempted through distant hybridization, delayed pollination, application of irradiated pollen, hormone treatments and temperature shocks. However, none of these methods are dependable. The development of numerous pollen plantlets in anther culture of *Datura innoxia*, first reported by two Indian Scientists (Guha and Maheswari) was a major breakthrough in haploid breeding of higher plants. This technique of haploid production through anther culture (anther androgenesis or simply androgenesis) has been extended to numerous plant species including cereals, vegetables, oil and tree species.

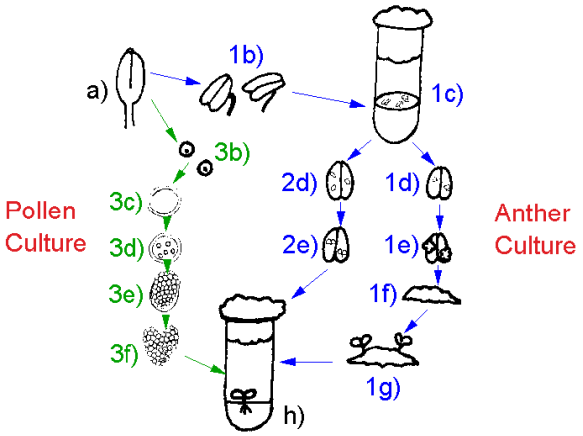
The anthers may be taken from plants grown in the field or in pots, but ideally these plants should be grown under controlled temperature, light and humidity. Often the capacity for haploid production declines with age of donor plants. Flower buds of the appropriate developmental stage are collected, surface sterilized and their anthers are

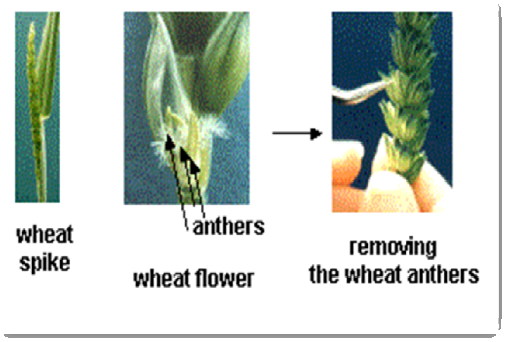
excised and placed horizontally on culture medium. Care should be taken to avoid injury to anthers since it may induce callus formation from anther walls. Alternatively, pollen grains can be separated from anthers and cultured on a suitable medium.

Pollen or Microspore culture

The pollen grains are released from the cultured anthers either mechanically. Or the cold treated anthers cultured on liquid medium burst open after 2-7 days liberating the pollen grains into the medium. This is called 'float culture method' which has proved better than mechanical isolation of pollen from fresh or pre-cultured anthers.

To improve the efficiency of isolated pollen culture for the production of haploids, Wenzel and his colleagues introduced the technique of density gradient centrifugation which allows the separation of embryogenic grains from a mixture of embryogenic and non-embryogenic grains obtained after crushing the anthers. The anthers of Barley obtained at the proper stage of development and gently macerated to obtain a suspension of pollen grains. After removing the debris by repeated filtration and centrifugation, the suspension was layered on 30% sucrose solution and centrifuged at 1200 g for 5 min. The androgenic, vacuolated pollen grains formed a band at the top of the sucrose solution. Isolated pollen culture is not only more efficient but also more convenient than anther culture. The tedious process of dissection of anthers is avoided. Instead, the entire buds within a suitable size range are crushed and the embryogenic grains are then separated by gradient centrifugation (Figure).





Pathways of development

The early divisions in responding pollen grains may occur in one of the following four ways (Figure).

1. Pathway I: The uninucleate pollen grain may divide symmetrically to yield two equal daughter cells both of which undergo further divisions. *(Dature innotura)*
2. Pathway II: In some other cases (*Nicotiana tabacum, Datura metel, Triticale)*, the uninucleate pollen divides unequally (as it does in nature). The generative cell degenerates immediately or after undergoing one or two divisions. The callus/embryo originate due to successive divisions of the vegetative cells.
3. Pathway III: But in some species like *Hyoscyamus niger,* the pollen embryos originate from the generative cell alone; the vegetative cell either does not divide or divides only to a limited extent forming a suspensor like structure.
4. Pathway IV: In certain species such as *Datura innoxia* the uninucleate pollen grains divide unequally, producing generative and vegetative cells, but both these cells divide repeatedly to contribute to the developing embryo/callus.

Pollen grains of many crop species, e.g. Tobacco, Wheat, Barley etc., exhibit pollen dimorphism. Most of the pollen grains are bigger, stain deeply with acetocarmine and contain plenty of starch. But small portions (ca. 0.7%) of the pollen grains are smaller and stain faintly with acetocarmine; these are called S-grains. These S-grains only respond during anther culture. The frequency of responding pollen grains can be enhanced over that of S-grains by certain pretreatments. E.g. chilling. Pollen grains of the cultured anthers show remarkable cytological changes during the first 6-12 days, called the inductive period. In tobacco, the gametophytic cytoplasm of binucleate pollen grains is degraded, ribosomes are eliminated and only few mitochondria and plastids remain. New ribosomes are synthesized following the first sporophytic division of the vegetative cell.

The responsive pollen grains become multicellular and ultimately burst open to release the cell mass. This cell mass may either assume the shape of a globular embryo and undergo the developmental stages of embryogeny or it may develop into callus depending on the species. Regeneration of plants from pollen callus or pollen embryos may occur on the original medium or it may require transfer to a different medium. The

pollen embryo exhibit considerable similarity with zygotic embryos in their morphology and certain biochemical features. Often the pollen embryos do not germinate normally. Pollen embryos frequently produce secondary embryos on stem surface and all such embryos which produce secondary embryos are haploid and the others non-haploid. To raise full plantlets from pollen embryos it is necessary to excise a cluster of the secondary embryos along with a part of the parent embryo and plant them on fresh medium. They do not germinate if left on the pollen embryo or removed individually.

Factors affecting androgenesis

1. Physiological status of the donor plants- The age of the donor plants and the environmental conditions under which it has been grown significantly affects the androgenic process. Generally, the buds from the first flush of flowers show better response than those borne separately. Exposures of donor plants to nutrient and water stresses reported to promote androgenesis.
2. Stage of pollen development- The pollen grains around the first mitosis is most responsive. The uninucleate microspores produce haploids while the binucleate pollen form plants of higher ploidy.
3. Anther wall factors- the pollen from one cultivar of tobacco would successfully develop into an embryo even if transferred into the anthers of another cultivar.
4. Genotype – hybrids are more androgenic than their parents.
5. Pretreatment of cultured anthers/pollen grains – application of certain physical (temperature shock, centrifugation, γ irradiation) and chemical (auxins) treatments to cultured anthers or pollen grains prior to standard culture room conditions, has proved essential or promotory for in vitro androgenesis.
6. Culture medium – addition of etherel (2-chloroethylphosphonic acid), sucrose, agar and other nutrients specific to certain genotype found to increase the success rate of androgenesis.
7. Culture density- the frequency of pollen embryogenesis was enhanced if the anther culture density was increased from 3 anthers per ml to 12-24 anthers per ml in *Brassica oleracea.*

8. Effect of gaseous environment- the composition of the gas mixture that surrounds the anthers has profound influence on the number of embryos produced in anther cultures. The removal of CO_2 from the culture vessel resulted in decline in anther culture response in *Nicotiana tobaccum.*
9. Effect of light – Isolated pollen cultures are more sensitive to light than anther culture.

Applications

1. Production of doubled haploids: Homozygous lines of the cross pollinating species and hybrids are highly desirable to increase the efficiency of selection and production of homozygous plants. The conventional method to produce homozygous plants is lengthy and laborious, requiring 7-8 recurrent cycles of inbreeding. Moreover, this approach is impractical for self-incompatible and male sterile and tree species. On the other hand, homozygous plants can be obtained in a single generation by diploidization of the haploid. This kind of production of stable, homozygous dihaploids (DH) in a single generation equivalent to the F_α generation of pedigree breeding and thus considerably shortens the breeding cycle. Generally, colchicines is recommended to diploidize the pollen plants. In practice, the pollen derived plants are immersed in filter sterilized solution of colchicines or applied as lanolin paste or injecting into the secondary buds or by root feeding. Besides bringing about chromosome duplication, colchicines treatment may also result in chromosome and gene instabilities. Therefore, the frequent occurrence of spontaneous duplication of chromosomes in differentiated plant cells (cortex, pith) and callus cells in long term cultures has also been exploited to raise homozygous fertile diploids from haploid plants (Figure). In this method, pieces of vegetative parts such as stem, root or petiole segments are cultured in a suitable medium to induce callusing. The initial callus may have some diploid cells but their frequency would increase in repeated subcultures. Such calli are transferred to the plant regeneration medium. Many of the plants so derived are diploid. However, the ploidy of individual plants must be confirmed before incorporating them in further experiments.

2. Normally, in a hybridization programme evaluation of lines is possible only after 4-5 years of backcrossing (F5 or F6 generations) and it takes another 4-5 years to release a new variety. By anther culture of F1 hybrids the various genotypes of gametes can be fixed and evaluated in the first generation. Anther culture can itself generate new recombinations and fix them simultaneously.
3. Haploids are extremely useful for detecting recessive mutants which may not express themselves in the heterozygous diploid background and therefore can be easily lost.
4. Gametoclonal variation –in vitro androgenesis provides a unique opportunity to screen the gametophytic variation caused by recombination and segregation during meiosis. For example, a gametoclone of tomato, which bears fruits with higher solid content than the parent cultivar, has been produced through anther culture.
5. *M*utagenesis- Detection and isolation of recessive mutants in the haploid state and rapid obtainment of the mutated gene in a homozygous diploid state is a special merit of haploidy in higher plants. Application of mutagenic treatment at the microspore stage, which is a single celled structure, has the added advantage of obtaining solid mutants. Through, microspore mutagenesis, a mutant of *Brassica napus* with high oleic and low lanoleic acid content was obtained.
6. Production of super male of *Asparagus officinalis*- In *A. officinalis*, a dioecious crop species, an inbred population is produced through sib crosses between pistillate and staminate plants which yield 50 % males and 50 % females. However, the commercially desirable features of this crop are uniform male population with spears having low fibre content. Anther culture was used to produce haploids of this species and this was diploidized to raise homozygous males. These are called as super-males.

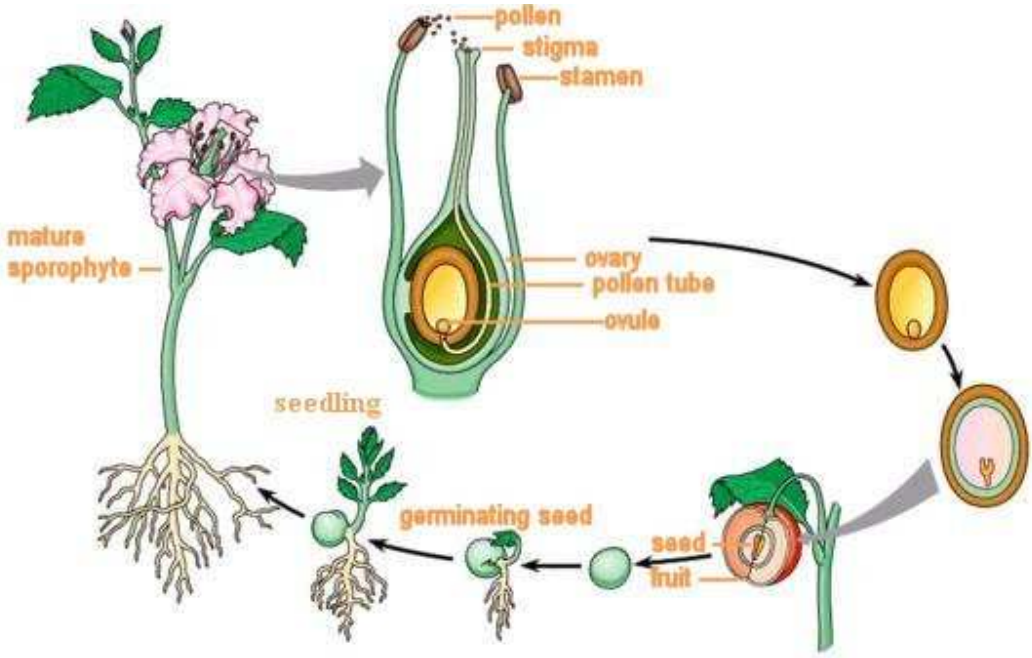
Limitations

1. Low Yield- generally 5-8% of the total pollen grains in a responding anther undergo androgenic development.

2. 70-80% of the embryos are incapable of normal germination due to structural, physiological and biochemical abnormalities of pollen.
3. Occurrence of high frequencies of albinos in cereals.
4. Instability of genetic material during androgenesis.

Ovary culture

Culture of unfertilized ovaries to obtain haploid plants from egg cell or other haploid cells of the embryo sac is called ovary culture and this process is termed as



gynogenesis. San Noem first reported the gynogenesis in barley in 1976. Subsequently, success has been obtained in several species including wheat, rice, maize, tobacco, sugar beet, rubber etc. About 0.2-6% of the cultured ovaries show gynogenesis and one or two, rarely up to 8, plantlets originate from each ovary. The rate of success varies considerably with species and is markedly influenced by the genotype so that some cultivars do not respond at all. E.g. In rice, *japonica* genotypes are far more responsive than *indica* genotypes.

In most cases, the optimum stage for ovary culture is the nearly mature embryo sac, but in rice ovaries at free nuclear embryo sac stage are the most responsive. Generally, culture of whole flowers, ovary and ovules attached to placenta respond better,

but in *Gerbera* and Sunflower isolated ovules show better response. Cold pretreatment (24-48 hr at 4°C in sunflower and 24 hr at 7°C in rice) of the inflorescence before ovary culture enhances gynogenesis.

Growth regulators are crucial in gynogenesis and at higher levels they may induce callusing of somatic tissues and even suppress gynogenesis. Growth regulator and sucrose requirement seems to depend on species. Ovaries are generally cultured in light, but at least in some species, e.g. sunflower and rice, dark incubation favours gynogenesis and minimizes somatic callusing; in rice light may lead to degeneration of gynogenic proembryos.

Generally, gynogenesis has two or many stages and each stage has distinct requirements. In rice, tow stages viz., induction and regeneration, are recognized. During induction, ovaries are floated on a liquid medium having low auxin and kept in dark, while for regeneration they are transferred on to an agar medium with higher auxin concentration and incubated in light. As in anther culture, gynogenesis may occur either via embryogenesis or through plantlet regeneration from callus. In general, regeneration from a callus phase appears to be easier than direct embryogenesis.

Advantages

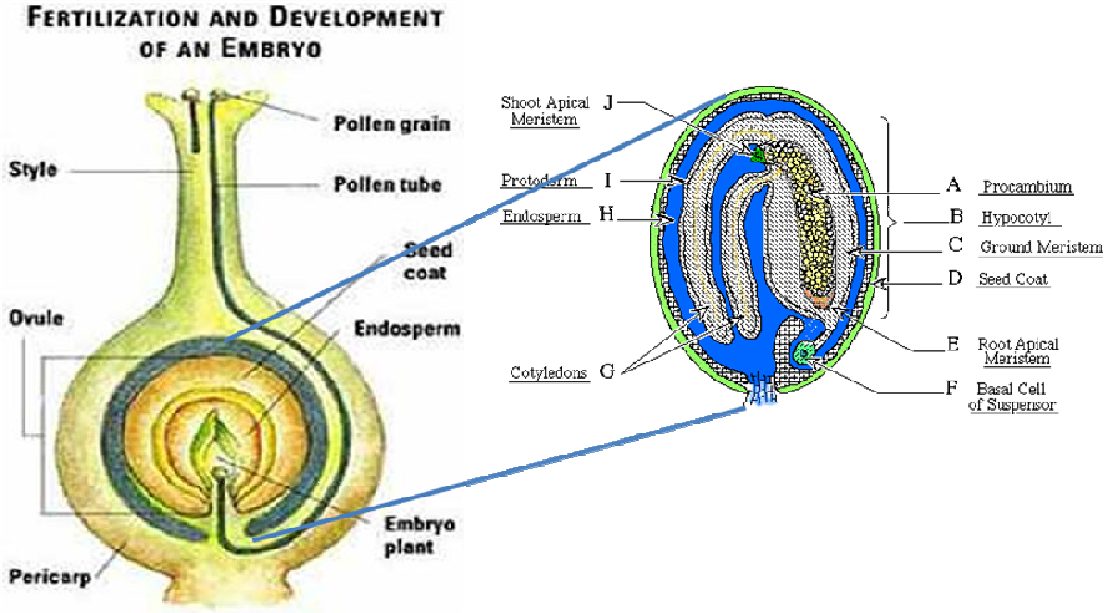
1. Gynogenetic haploids may be a valuable substitute for the production of homozygous lines in cases where cytoplasmic male sterility prevents the use of micropsores.
2. Reduction in the frequency of albino plants in some species especially cereals.

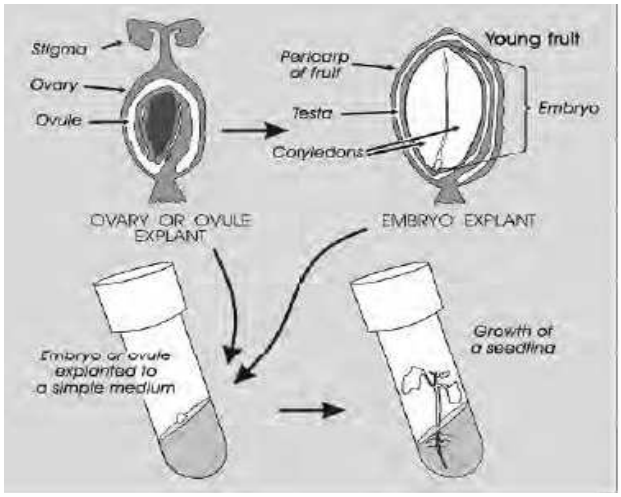
Limitations

1. So far it has been successful only in less than two dozens species.
2. The frequency of responding ovaries (1-5%) and the number of plantslets/ovary (1-2) I quite low. Therefore, anther culture is preferred over ovary culture. Only in those cases where anther culture fails, e.g. sugarbeet and for male sterile lines, ovary culture assumes significance.

Embryo culture

Embryo culture is the sterile isolation of an immature or mature embryo *in vitro* with the goal of obtaining a viable plant.





Types

a. Culture of immature embryos originating from unripe seeds, that is mainly to avoid embryo abortion with the purpose to produce a viable plant.

b. Culture of mature embryos derived from ripe seeds.

Factors affecting the success of embryo culture

1. Genotypes
2. Developmental stage of the embryo at isolation
3. Growth conditions of the mother plant
4. Composition of the nutrient media
5. Light
6. Temperature

Practical applications

1. Elimination of inhibitors of seed germination
2. Shortening breeding cycle
3. Overcoming self sterility of seeds e.g. *Musa bulbisiana* and tubers crops: *Colocasia esculenta & C. antiquorum.*
4. Seed Testing: Rapid means of determining viability of particular lot of seeds eg. seeds of conifers, shrubs, vines and fruit trees.
5. Prevention of embryo abortion in early ripe fruits e.g. Peach, cherry, apricot, plum.
6. Prevention of embryo abortion as a result of incompatibility (embryo rescue). E.g. In interspecific (*Phseolus*, lily flex, cotton, tomato, rice and barely), intergenic (Hordeum x Seale and Triticum x Seale.) crosses and crosses between diploids and tetraploids (barley and Rye).
7. Vegetative propagation. E.g. In Gramineae and Coniferae embryos are often used as a starting material.
8. **Other applications** To study fundamental problems in experimental embryogenesis. Host pathogen interaction. *e.g*. formation of ergot by infection of rye (*Phaseolus vulgaris)* embryos by *claviceps purpurea* and fusarium wilt of seedlings. In latter case, incorporation of fungal toxin fusaric acid into culture medium interfere with water uptake by germinated embryos of rye and induce characteristics wilting of embryonic leaves. Cultured embryos have been used as test objects to evaluate the mutagenic ability of irradiated substrates on living

tissues. For this embryos of certain cereals were planted on X-irradiated nutrient medium for evaluation.

Embryo rescue

Distant crosses may fail due to one or more of several reasons such as inability of pollen to germinate, failure of pollen tubes to grow or perhaps more commonly degeneration of endosperm. When embryo fails to develop due to endosperm degeneration, embryo culture is used to recover hybrid plants. This is called as hybrid rescue through embryo culture. Some recent examples are the recovery of hybrids from *Hordium vulgare X Secale cereale, Triticum aestivum X Agropyron repens, H. vulgare X Triticum aestivum etc.,* In case of *Triticale* rare combinations between *Triticale and Secale* develop viable seeds. But most of the tetraploid and hexaploid wheat carry two dominant genes, *Kr1* and *Kr2,* which prevent seed development in crosses with *Secale*. The majority of the hybrid seeds are small, poorly developed and show very poor germination. Further, seeds are obtained from only 5-10% of the florets pollinated. The recovery of hybrid seedlings is much greater (50-70%) when embryos from 10-14 day old caryopses are removed and cultured on a suitable medium.

Bulbosum technique

Principle

The fertilization proceeds readily between *H. vulgare* and *H. bulbosum.* Zygote induction is high and chromosomes of *H. bulbosum* are rapidly eliminated from the cells of developing embryo. This develops for two to five days and then aborts. In the developing monoploid embryo cells, the division and increment is slower than the diploid cells. This comparatively slow growth of the monoploid condition, together with the disintegration of the endosperm leads to the formation of small embryos which have to be dissected out of the fruits and provided with nutrients *in vitro* in order to complete their development. Following *in vitro* embryo culture, the developing plantlets are raised under normal green house conditions and chromosome doubling is induced on established plants.

Advantages

1. The method of hybridization followed by chromosome elimination proves to be of general interest for haploid production in other species of *Hordeum* and also of hexaploid wheat.
2. It is possible to produce monoploids of barely in a cytoplasm of *H. bulbosum* by using *H. vulgare* as male and *H. bulbosum* as female. Using embryo culture as vehicle, high frequency foreign cytoplasm monoploids can be obtained.
3. *Hordeum* species is not the only one where chromosome elimination is found in higher plants. In Haplopoppus, monoploids have been examined with only two chromosomes. *H. bulbosum* need not be the ideal partner for *H. vulgare* to induce monoploids of barley via somatic chromosome elimination. There can be a range of *Hordeum* that might be tried as a more efficient pattern than *H. bulbosum*.

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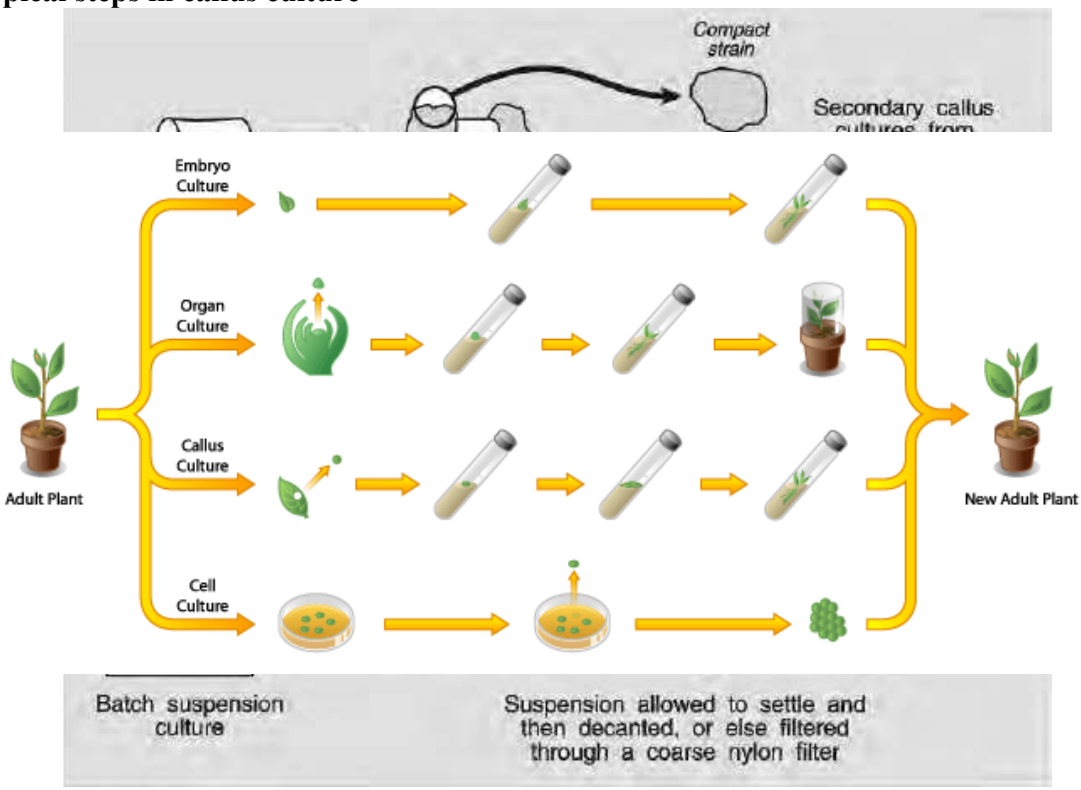
Lecture.31

Callus and Cell cultures. Somaclonal Variation

Callus Culture

Callus consists of an amorphous or undifferentiated mass of loosely arranged thin-walled parenchyma cells arising from the proliferating cells of the parent tissue. For callus initiation, transfer the explants aseptically to the semi solid medium and gently press them into the agar in such a way that good contact is made. Radical tips are laid horizontally on the agar whereas stem sections may be placed vertically with one cut end in the agar, to confer good callus. Within 2-3 weeks, explants should show new growth as pustules or protuberances or as a fine mat across the surface depending on the distribution and mitotic activity of the parenchyma residing in the excised tissues. Some callus growths are heavily lignified and hard in texture whereas others break easily into small fragments. Most explants produce sufficient callus in an appropriate medium to allow for subculture within 3-7 weeks. When cultured for several weeks, callus will show signs of ageing. This can be noted as deceleration of growth, necrotic or browning and finally desiccation. The newly formed callus will be removed from the initial explant at this stage by cutting with the sterile scalpel. Once well established, most callus cultures will require regular subculture at approximately 4 weeks interval.

Typical steps in callus culture

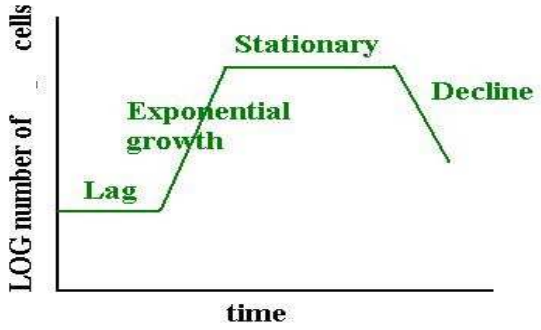


Cell suspension culture

To obtain free cells, pieces of undifferentiated and friable calli are transferred to liquid medium in flask or some other suitable vial and the medium is continuously agitated by a suitable device. Such cultures is called 'suspension culture'. Agitation of medium serves at least two functions. First, it exerts a mild pressure on cell aggregates, breaking them into smaller clumps and single cells. Second, the agitation maintains uniform distribution of cells and cell clumps in the medium. Movement of the medium also provides gaseous exchange between the culture medium and culture air.

Most suspension cultures are obtained by transfer of friable callus clumps to agitated liquid medium of the same composition as that used for callus growth. Agitation rates on orbital shakers should be in the range of 100-150 rpm. The degree of cell separation of established cultures of high friability can be modified by changing the composition of the nutrient medium, particularly the concentration of growth regulators.

When the plant material is first placed on the medium, there is an initial lag period prior to any sign of cell division (Figure).



During the incubation period, the biomass of the suspension cultures increases due to cell division and cell enlargement. This continues for a limited period after which the growth stops due to the exhaustion of some factors or the accumulation of certain toxic metabolites in the culture medium. When a small aliquot of the cell suspension at this stage, is transferred to a fresh medium of the same composition, the cell growth is revived. This is an exponential rise in cell number and a linear increase in cell population. There is a gradual deceleration in the division rate. After three to four cell generations the growth declines and finally the culture enters the stationary phase. Finally the cells enter a stationery or non dividing stage. In order to maintain the viability of the culture, the cells should be sub cultured early during this stationery phase i.e., in exponential phase by frequent (every 2-3 days) subculture of the suspensions. Prolonged maintainance of cultures in the stationary phase may result in extensive death and lysis of cells.

Subculture involves the aseptic transfer of a suitable volume of inoculum to fresh medium using either pipettes (or) autoclavable metal syringes and transfer by simply tipping culture into the new vessel up to a graduation mark to ensure approximately constant inoculum's size. At the time of subculture, the flask is allowed to stand for a few seconds to allow the large colonies to settle down and suspension is taken from the upper part of the culture. Regular practice of this procedure should allow the build-up of a fine suspension.

The texture of a callus is genetically controlled. Certain celli may not give good dispersion under normal media conditions. However, it has been possible to improve the tissue dissociation by manipulating the media composition and subculture routine. Addition of small amounts of 2, 4-D, hydrolytic enzymes such as cellulose and pectinase or substances like yeast extract had a promotory effect on cell dispersion.

Subculture Methods

Basically there are three types of suspension cultures: batch culture, continuous culture and immobilized culture.

Batch culture

These are used for initiating single cell cultures. Cell suspensions are grown in 100-250 ml flasks each containing 20-75 ml of culture medium. The cultures are continuously propagated by routinely taking a small aliquot of the suspension and transferring it to a fresh medium (ca. 5X dilution). Batch cultures are not ideal for studies of cell growth and metabolism because there is a constant change in cell density and nutritional status of the medium. In these cultures the exponential growth with constant cell doubling time may be achieved for a short time but there is no period of steady-state growth in which the relative cell concentrations of metabolites and enzymes are constant. To a certain extent these problems are overcome by continuous cultures.

Continuous culture

In a continuous culture, the cell population is maintained in a ~fixed number by regularly replacing a portion of the used or spent medium by fresh medium. Such culture systems are of either closed or open type. In a *closed continuous culture*, cells are separated from the used medium taken out for replacement and added back to the culture so that cell biomass keeps on increasing. Whereas in an *open continuous culture,* both cells and the used medium are taken out and replaced by equal volume of fresh medium and cells. The replacement volume is so adjusted that cultures remain at sub-maximal growth indefinitely.

The open cultures are of either turbidostat or chemostat types. In a *turbidostat,* cells are allowed to grow up to a preselected turbidity (usually measured as OD) when a predetermined volume of the culture is replaced by fresh normal culture medium. But in a *Chemostat,* a chosen nutrient is kept in a concentration so that it is depleted very rapidly to become growth limiting, while other nutrients are still in concentrations higher than required. In such a situation, any addition of the growth limiting nutrient is reflected in cell growth. Chemostats are ideal for the determination of effects of individual nutrients on cell growth and differentiation.

Immobilized cell cultures

Plant cells and cell groups are encapsulated in a suitable compound such as agarose, calcium alginate cells etc. or entrapped in membranes or stainless steel screens. The gel beads containing cells may be packed in a suitable column or alternatively, cells may be packed in a column of a membrane or wire cloth. Liquid medium is continuously run through the column to provide nutrients and aeration to cells. Immobilization of cells changes their cellular physiology in comparison to suspension culture cells. This offers several advantages for their use in biochemical production, but they are generally not used for other studies.

Estimation of Growth

The growth of cell suspension cultures may be monitored by measurement of one or more of the following parameters.

1. Cell number is the most informative measure of cell growth. This measurement is applicable to only suspension cultures and even there cell aggregates must be treated with pectinase, to dissociate them into single cells before counting the cell number in a **haemocytometer.**

2. **Packed cell volume** of suspension cultures is easily determined by pipetting a known volume in to a 15 ml graduated centrifuge tube, spinning at 2000 rpm for 5 minutes and reading the volume of cell pellet which is expressed as ml cells/l of culture.

3. Culture fresh and dry weights are the most commonly used measures of growth of both suspension and callus cultures. In case of callus cultures, the cell mass is

placed on a preweighed dry filter paper or nylon filter and weighed to determine fresh weight. Cells from a suspension culture are filtered on to a filter paper or nylon filter, washed with distilled water, excess water removed under vacuum and weighed along with the filter; the filter is preweighed in wet condition. For dry weight determination, the cells and the filter are dried in an oven at 60°C for 12 hr and weighed. The filter is preweighed in dry condition. Cell fresh and dry weights may either be expressed as 'per ml or per culture'.

4. Medium conductivity- A Conductivity change of the culture medium is inversely proportional to cell fresh weight.

5. Cell viability- In addition to microscopic examination for protoplast streaming and the presence of an intact nucleus, cell viability may also be assessed by use of vital staining of intact living cells eg. Evan's blue (0.025% w/v) or colored salts (Tetrazolium) or Fluorescein diacetate - which are metabolized in living cells to give fluorescent products.

6. Mitotic Index is the ratio between Number of Nuclei in mitosis and Total number of nuclei examined in the sample.

***In vitro* Secondary metabolites production**

Secondary metabolites are those compounds which are not directly involved in the primary metabolic processes such as photosynthesis, respiration, protein and lipid biosynthesis etc. Secondary metabolites include a wide variety of compounds such as alkaloids, terpenoids, phenyl propanoids etc. These substances do not participate in vital metabolic functions of the host plant tissues in the same manner as amino acids, nucleic acids or other primary metabolites but appear to serve as a chemical interface between the plant and its surrounding environment. These are used to protect against pests and to attract pollinators. They may also help in combating infectious diseases.

Higher plants are valuable sources of industrially important natural products which include flavors, fragrances, essential oils, pigments, sweeteners, feed stocks, anti microbial and pharmaceuticals (about 25 % of the prescribed medicines are solely derived from plants) (Table 1). Many of the pharmaceutical compounds have complex

structures which makes their chemical synthesis economically unattractive. Cultured cells of many plant species produce novel biochemicals which have so far not been detected in whole plants. For example, cell suspension cultures of *Rauwolfa serpentine* have been shown to produce highly polar alkaloids which are novel glucosides of ajmaline and its derivatives.

Extraction of certain secondary metabolites from cell lines has definite advantages.

 Plant cells are relatively easy to grow.

 The rate of cell growth and biosynthesis of secondary metabolites under *in vitro* cultures is quite high and the product may be produced in a short period of time.

 Plant cell cultures are maintained under controlled environmental and nutritional conditions which ensure continuous yields of metabolites.

 Suspension cultures offer a very effective way of incorporating precursors into cells

 New routes of synthesis can be recovered from deviant and mutant cell lines which can lead to production of novel compounds which are not previously found in whole plants.

 Some cell cultures have the capacity for bio transformation of specific substrates to more valuable products by means of single or multiple step enzyme activity.

 Culture of cells may be more economical for those plants which take long period to achieve maturity.

Disadvantages

1. High production cost
2. Lack of basic knowledge on the biochemical pathways.
3. Often cultured plant cells do not produce compounds of high value.

Improvement of secondary metabolite production through cultured plant cells

Biochemical yields from cultured plant cells can be improved by increasing cell biomass yield per unit volume of culture and biochemical content of the cell biomass.

1. **Enhancement of Biomass yields**

Most of the biochemicals are produced in differentiated cells or organized tissues. Therefore, these biochemicals can not be produced in rapidly growing cell cultures and the culture conditions favoring growth suppress biochemical production and *vice-versa.* Hence, the production strategy consists of two phases: i. Growth phase- for cell biomass accumulation ii. Production phase - for biosynthesis and accumulation of the biochemicals. Biomass accumulation can be improved by using optimum culture conditions of which nutrient medium and inoculum size are particularly important. For example, *Lithospermum erythrorhizon* suspension culture yielded Shikonin of only 6.8 g cell dry weight/l on White's medium, while the yield on LS (Linsmaier and Skoog) medium was 16.8 g/l. Biomass production can be markedly increased by the use of a larger inoculum size, to give higher initial cell density, in combination with proportionately enriched nutrient medium. For example, twice the concentration of normal medium for a two-fold increased inoculum size.

2. **Improving Biochemical production**

Biochemical production by cultured cells can be increased by;

- Devising a suitable culture medium and conditions

  E.g. Auxins, especially, IAA increase the Shikonin production by *Lithospermum* cells.

- Development of high producing cultures

  All the clones of *Lithospermum* seedlings did not produce Shikonin. Hence, it is necessary to identify high producing clones among the several clones produced by the single genotype.

3. **Use of elicitors**

The molecule which stimulates the synthesis of secondary metabolites are called as elicitors (the phenomenon is called as elicitation). Eg. Biotic elicitors : 1. Endogenous elicitors- pectins, cellulose etc., 2. Exogenous elicitors – chitin, glucans etc. (microbial origin). Abiotic elicitors such as UV light, salts of heavy metals and chemicals that disturb membrane integrity can also be used. Cell wall pectins (100 mg/l of medium),

increased anthraquinone production by *Morinda citrifolia* to 5.6 times that of control after 14 days incubation.

4. **Use of organ cultures**. For example, the monoterpenes in *Mentha* spp. are synthesized and stored in epidermal oil glands of leaves. Shoot cultures of *Mentha* grown on growth regulator free medium accumulate monoterpenes. In some cases, the secondary metabolites may be released in the medium, where they may be degraded or their synthesis may be regulated (suppressed) by feedback inhibition. In this case, the biochemicals are removed from the medium by adding certain compounds which absorbs (Charcoal), dissolves (Miglyol) or encapsulates (β-cyclodextrin). Such a culture system is called as two phase system. The culture medium constitutes one phase, while the adsorbant compound makes the second phase.

Commercial Production of Shikonin

Shikonin is the first commercial product from cell cultures. The high producing clone cells to be used as inoculum (Stock or seed cultures) are maintained in jar fermenter. The inoculum is then added to 200 l fermenter (first stage) containing the MG-5 medium for culture growth. After 9 days, the cells are filtered out and inoculated into a 750 l fermenter (second stage) containing M-9 shikonin production medium and incubated for 14 days. The cells are harvested by simple filtration and Shikonin and Shikonin derivatives are extracted from the cells. A 700 l bioreactor with 600 l medium would yield 1.2 kg of Shikonin in 2 weeks.

Hairy root cultures

Agrobacterium rhizogens may be used to transform leaf discs, other organs or even protoplasts. This induce hairy roots which may arise directly from that site of transformation. These hairy roots synthesize secondary metabolites at the levels characteristic of the roots of parent plants. The roots are excised and used to initiate root cultures and it is easily developed in most dicots. Hairy root cultures are

1. Suitable for production of all root-derived biochemicals

2. faster growing, higher producing and much easier to maintain than untransformed root cultures
3. do not have risk of instability which is common is cell cultures

Biotransformation

Modification of an exogenous compound by plant cells (or other biological organisms) is called biotransformation or bioconversion. These reactions (esterification, oxidation, reduction, hydroxylation and glycosilation etc.,) are catalyzed by enzymes present in the plant cells. For example, cell cultures of *Datura innoxia, Catharanthus roseus, Rauwolfia serpentina* biotransform hydroquinone into its β-D-glucoside called arbutin. Arbutin is an efficient suppressor of melanin biosynthesis in human skin and so used in cosmetics.

Table 1. Selected examples of secondary metabolites produced by plant species.

Compound	Class of compound	Plant species	Yield (g/l)	Use of the compound
Shikonin	Napthoquinones	*Lithospermum erythrorhizon*	4	Antiseptic; dye for silk and cosmetics
Berberine	Alkaloid	*Coptis japonica*	7	Antibacterial and anti-inflammatory
Taxol	Diterpene alkaloid	*Taxus* spp.	-	Breast and ovarian cancer treatment
Ajmalicin	Alkaloid (indole alkaloid)	*Catharanthus roseus*	2	Antileukaemic

Somaclonal variation

It is the term used to describe the variation seen in plants that have been produced by plant tissue culture. Chromosomal rearrangements are an important source of this variation.

Origins and mechanisms of somaclonal variability

Such variation was thought to be due to epi genetic factor such as exposure to plant growth regulators (PGRs) and prolonged culture time

Somaclonal variation can be of two sorts:

- Genetic (i.e. heritable) variability – caused by mutations or other changes in DNA.

- Epigenetic (i.e. non-heritable) variability – caused by temporary phenotypic changes.

Genetic variability

Various molecular mechanisms are responsible for genetic variability associated with somaclonal variation:

1. Changes in ploidy
2. Structural changes in nuclear DNA
3. Epigenetic variability

The importance of somaclonal variation

1. A novel source of genetic Variation. However successful utilization of somaclonal variation heavily depends upon its systematic evaluation and judicious utilization in breeding programmes. E.g. scented geranium viz., velvet rose; pure thronless blackberries – Lincoln logon (Rubus); Hasuyume – protoplast derived rice cultivar; potato – Russet barbank
2. It is of interest as a basic genetic process, since it contradicts the concept of clonal uniformity.
3. Third soma clonal variation is unwanted when the objective is micropropagation of elite genotypes or genetic transformation that partly involved tissue culture. Under such circumstances, prevention or at least minimization of variation is of utmost importance. To achieve this, the frequency, nature and magnitude of somaclonal variation in relation to manipulation of media components, explant source, culture conditions etc. should essentially be understood.
4. Somaclonal variation are employed in disease resistance improvement. For example, in sugar cane, it was used to develop eye spot disease resistance caused by *Helmenthosporea sacchari*, downey mildew, and fizi virus; in potato, protoplast derived russet bar bank resistant to phytopthora infestans (Late blight of potato).
5. Somaclonal variation can also be used to impart abiotic stress resistance such as drought, salinity, aluminium, herbicide tolerance and insect resistance.

Why variation occurs?

Factors that contribute to soma clonal variation are of categories i.e. physiological genetic and biochemical.

Physiological causes of variation

Variations induced by physiological factors were identified quite earlier. Such variations are those induced habituation to PGR in culture and culture conditions and are epigenetic. They may not be inherited in Mendelian fashion. Prolonged exposure of explant tissue to powerful auxin such as phenoxyacetic acid **(e.g., 2, 4-D or 2, 4, 5-T)** often results in variation among the regenerants.

Genetic causes of variation

Tissue culture plants show certain variations which are results of alteration at the chromosomal level. Although the explant tissue may be phenotypically similar, plant often have tissue comprised of diverse cell type or cells. That is there are cytological variation among the cell types within the explant tissue, such pre existing conditions often result in plant regenerates from the tissue that are dissimilar.

Biochemical cause of variation

Biochemical variations are predominant type of variations in tissue culture. Biochemical variations include alteration in carbon metabolism leading to lack of photosynthetic ability (albinos in cereals such as rice), starch biosynthesis carotenoid pathway.

Selection of somaclones

Although cytological and phenotypic analyses can be used to evaluate somaclonal variation, recently molecular techniques (such as RFLP, RAPD, SSR, AFLP) have been used with increasing frequency.

Benefits

The major likely benefit of somaclonal variation is in plant improvement. Somaclonal variation leads to the creation of additional genetic variability. Characteristics for which somaclonal mutants can be enriched during *in vitro* culture include resistance to disease or patho- toxins, herbicides and tolerance to environmental or chemical stress, as well as for increased production of secondary metabolites.

Disadvantages

A serious disadvantage of somaclonal variation occurs in operations which require clonal uniformity, as in the horticulture and forestry industries where tissue culture is employed for rapid propagation of elite genotypes.

Ways of reducing somaclonal variation

It is well known that increasing numbers of subculture increases the likelihood of somaclonal variation, so the number of subcultures in micropropagation protocols should be kept to a minimum. Regular re-initiation of clones from new explants might reduce variability over time. Another way of reducing somaclonal variation is to avoid 2,4-D in the culture medium, as this hormone is known to introduce variation. Vitrification [hyperhydracity] may be a problem in some species. In case of forest trees, mature elite trees can be identified and rapidly cloned by this technique. High production cost has

limited the application of this technique to more valuable ornamental crops and some fruit trees.

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Lecture.32
Protoplast: Isolation and Fusion

Protoplast Isolation

In eukaryotes the transfer of genetic material form one individual to another is conventionally achieved through sexual breeding. In plants, where fairly distant species could be crossed, it has not always been possible to obtain full hybrids between desired individuals because of sexual incompatibility barriers. In this respect cell fusion offers a novel approach to distant hybridization through somatic hybridization. Fusion of cells must occur through the plasma membrane. Unlike animals, in plants the plasma membrane is bound by a rigid cellulosic wall and the adjacent cells are cemented together by a pectin rich matrix. That's why somatic cell genetics is more advanced in animals than plants. In 1960, E. C. Cocking demonstrated the feasibility of enzymatic degradation of plant cell walls to obtain large quantities of viable cells – called as protoplasts.

Besides being able to fuse with each other, higher plant protoplasts can also take up foreign DNA, through their naked plasma membrane under specific chemical and physical treatments. Protoplasts also provide an experimental system for a wide range of biochemical and molecular studies ranging from investigations into the growth properties of individual cells to membrane transport.

Isolation of protoplast

1. Mechanical method

Klecker in 1892, has first initiated the protoplast isolation by mechanical means- the cells were kept in a suitable medium plasmolyticum and cut with a fine knife. In this process some of the plasmolyzed cells were cut only through the cell wall, releasing intact protoplasts.

Limitation:

- applicable only to vacuolated cells
- yields are extremely low.

2. Enzymatic method

In 1960, Cocking used a concentrated solution of cellulase enzyme, prepared from cultures of the fungus, *Myrothecium verrucaria,* to degrade the cell walls.

However, real progress in this area was made after 1968 when cellulase and macerozyme enzymes became commercially available. The commercial preparations of the enzymes for protoplast isolation were first employed by Takebe *et al*., (1968). The tobacco leaf species were first exposed to macerozyme to liberate single cells which were then treated with cellulase to digest the cell walls and release the protoplasts. Later, these two enzymes were used together and this is found as faster method and also reduces the chances of microbial contamination by cutting down a few steps.

A range of enzyme preparations are now available commercially (Table) and depending on the nature of the tissue these are used in different combinations. The use of commercially available enzymes has enabled the isolation of protoplasts from virtually every plant tissue as long as cells have not acquired lignification. Protoplast isolation has been reported from mesophyll cells of in vivo and in vitro growing plantlets (Figure), aseptic seedlings, microspore mother cells, young microspores, pollen grain calli and embryogenic and non-embryogenic suspension cultures. More recently, viable protoplasts have been obtained from male and female gametes.

Factors affecting yield and viability of protoplasts

1. Source of material: Leaf has been the most favorite source of plant protoplasts because it allows the isolation of a large number of relatively uniform cells without the necessity of killing the plants. Since the mesophyll cell are loosely arranged, the enzymes have an easy access to the cell wall. The leaves from in vitro roots or shoots released twice as many viable protoplasts as the leaves from field grown material. Owing to the difficulty in isolating culturable protoplasts from leaf cells of cereals and other species, their cultured cells have been used as an alternative source material.
2. Physiological state of the tissue and cell material: younger the tissue, more the success of protoplasts isolation.

3. Pre enzyme treatment: The lower epidermis is peeled and float the stripped pieces of leaf on the enzyme in manner that the peeled surface is in contact with the solution. This will facilitate easy penetration of enzyme in to intercellular spaces of leaf. Mesophyll protoplasts of cereals could be isolated within 2 hr by infiltrating the leaf pieces with enzyme solution under a partial vacuum for 3-5 min. The criterion used to check adequate infiltration is that leaf pieces will sink when the vacuum is removed. Brushing the leaf with a soft brush or with the cutting edge of scalpel may also improve enzymatic action.
4. Enzyme treatment: The two enzymes, essential to isolate protoplasts from plant cells are cellulase and pectinase. Pectinase degrades mainly the middle lamella and the cellulase required to digest the cellulosic cell wall. The crude commercial enzymes carry nucleases and proteases as impurities which may be harmful to protoplasts viability. The activity of the enzymes is pH dependant and it is also affected by the temperature. The optimal temperature for the activity of these enzymes is 40-50°C which happens to be too high for the cells. Generally 25-30°C is found adequate for isolation of protoplasts.
5. Osmoticum: A fundamental property of isolated protoplasts is their osmotic fragility and hence, there is a need for a suitable osmotic stabilizer in the enzyme solution, the protoplast medium and the protoplast culture medium. Protoplasts are more stable in a slightly hypertonic rather than isotonic solution. a higher level of the osmoticum may prevent bursting and budding but it may inhibit the division of the protoplast. The most widely used osmotica are sorbitol and mannitol in the range of 450-800 mmol.

Purification of protoplasts

After the material has been incubated in enzymes solution for an adequate period the incubation vessel is gently swirled or the leaf pieces are gently squeezed to release the protoplasts held in the original tissue. The digestion mixture consist of subcellular debris, especially chloroplasts, vascular elements, undigested cells and broken protoplasts besides intact and healthy protoplasts. The large debris is removed by passing through a metal or nylon filter (30-100 μm) and further purified by centrifugation.

Viability of the protoplasts

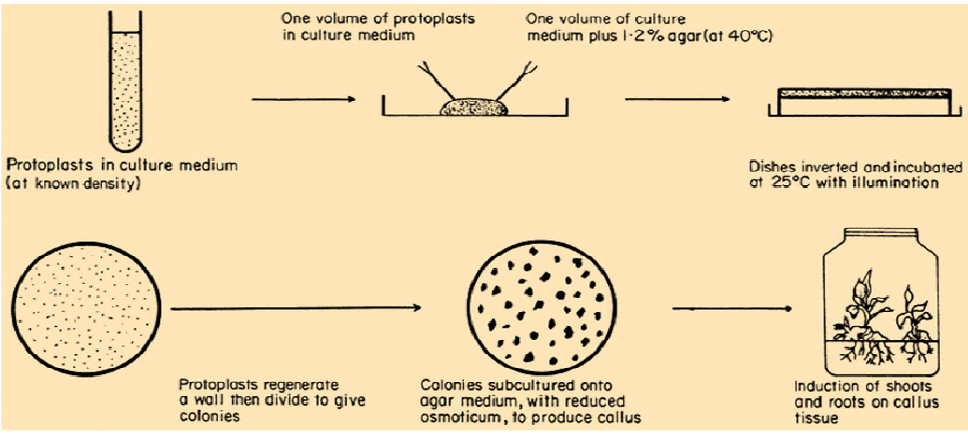
Viability of the freshly isolated protoplasts can be checked by a number of methods:

1. Observation of cyclosis or cytoplasmic streaming as an indication of active metabolism.
2. Oxygen uptake measured by an oxygen electrode which indicated respiratory metabolism.
3. Photosynthetic activity
4. Exclusion of Evan's blue dye by intact membranes
5. Staining with fluroscein diacetate- which is most commonly used.

Protoplast culture

Protoplasts may be cultured in agar plates. An advantage in using semi-solid medium is that the protoplasts remain stationary which makes it convenient to follow the development of specific individuals (Figure). However, liquid medium has been generally preferred for the following reasons:

1. the osmotic pressure of the medium can be effectively reduced after a few days of culture
2. protoplasts of some species would not divide if plated in agarified medium.
3. if the degenerating component of the protoplast population produces some toxic substances which could kill the healthy cells it is possible to change the medium.
4. the density of cells can be reduced or cells of special interest may be isolated after culturing them for a few days at a high density.



The protoplasts suspension is plated as a thin layer in petriplates, or incubated as static cultures in flasks or distributed in 50-100 µl drops in petri plates and stored in a humidified chamber. Embedding protoplasts in agarose beads or discs is reported to improve plating and regeneration efficiency in many species. Alginate is another gelling agent used for culture of protoplasts, particularly of the species which are heat sensitive such as *Arabidopsis thaliana.* After 2-4 days in culture, protoplasts lose their characteristic spherical shape and this has been taken as an indication of new wall regeneration. While the presence of a proper wall is essential for regular division, not all such cells regenerated from protoplasts embark upon division. In protoplast cultures, the cell divisions are asynchronous. The first division may be equal or unequal. Mitosis is normal. Continuous cell division leads to callus formation and the plant will be regenerated through normal developmental process.

Applications

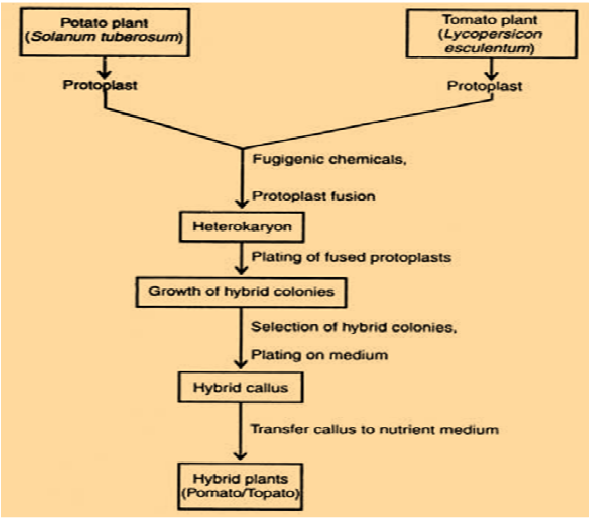
1. **Virus uptake:** Studies on the mechanism of infection and host parasite relationships
2. **Bacterial uptake:** Symbiotic nitrogen fixing bacterium (Rhizobium, Azotobacter) can be introduced into legume. Direct DNA transfer and expression of a bacterial gene in protoplasts of exogenous DNA by cells or protoplasts of *T. Monococcum* and *N. tabucum* are reported.
3. **Incorporation of Cyanobacterial cells** *(e.g.):* Cyanobacteria or BGA. Co incubate algal preparation with isolated protoplasts with 25% PEG and high planting density. Protoplasts begin engulf algal cells. **Incorporation of exogenous DNA:** Exogenous DNA can be taken up by higher plant cells/protoplasts and this is known as Trasngenosis.
4. **Transplantation of nuclei:** Organelles such a large nuclei can be introduced through plasma lemma into protoplasts. Both intra and inter specific nuclear transplantations have been observed in *Petunia hybrida, Nicotiana tabacum* and *zea mays*.

Table: Some commonly used commercially available enzymes for protoplast isolation

Enzymes	Source
Cellulases	
Onozuka RS Cellulase R-10	*Trichoderma viride* *T. viride*
Hemicellulase	
Hemicellulase Rhozyme HP150	*Aspergillus niger* *A. niger*
Pectinase	
Macerozyme R-10 Macerase	*Rhizopus* spp. *Rhizopus* spp.

Protoplast fusion

With the development of techniques for enzymatic isolation of protoplasts and subsequent regeneration, a new tool of genetic manipulation of plants has now become available. Moreover, the fusion of protoplasts of genetically different lines or species has also been possible. For example, some plants that show physical or chemical incompatibility in normal sexual crosses may be produced by the fusion of protoplasts obtained from two cultures of different species. (Somatic hybridization of crop plants represents a new challenge to plant breeding and crop improvement. In the field of pest and disease resistance and transfer of C_3 photosystems into C_4 crop plants somatic crosses show most interesting promises



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Lecture.33

Cryopreservation

In vitro germplasm conservation

With respect to seed preservation possibilities, plant species have been divided into 2 categories:

1. Orthodox seeds, which can withstand dehydration to *5%* or less (dry weight basis) without damage. When dry, the viability of these seeds can be prolonged by keeping them at the lowest temperature and moisture possible.
2. Recalcitrant seeds, which are high in moisture and are unable to withstand much desiccation. They are predominantly seeds from tropical or subtropical species. They can be stored only in wet medium in order to avoid dehydration injury and in relatively warm conditions because chilling injury is very common among these species. They remain viable only for a short time (weeks or months), even if kept in the required moisture conditions (e. g., oil palm, coconut, cacao coffee.

Moreover, there are practical problems in applying long-term seed storage to most long-live forest trees, including gymnosperms and angiosperms, since their juvenile period is very long and they do not produce seeds for several years. The conservation of plants which are vegetatively propagated, such as cassava, potato and yams also possess considerable problems.

In situ conservation has been made almost impossible due to the disappearance of large wild areas. Conservation *ex situ* is very difficult to carry out due to the following problems: an adequate sample has to be taken for the conservation of genetic diversity.

During the last years, *in vitro* culture techniques have been extensively developed and applied to more than 1,000 species, including many tropical species. The use of *in vitro* tissue culture techniques can be of great interest for germplasm collection, storage and multiplication of recalcitrant and vegetatively propagated species. Plant species that

are in danger of being extinct can be conserved using tissue culture techniques. Tissue culture systems present several advantages including:

(1) very high multiplication rates

(2) aseptic system:

-free from fungi, bacteria, viruses (after thermotherapy and indexation) and insect pests

-production of pathogen-free stocks

(3) reduction of space requirements ,

(4) genetic erosion reduced to zero under optimal and

(5) reduction of the expenses in labour costs

Further, tissue culture systems greatly facilitate the international exchange of germplasm. Indeed, the size of the samples is drastically reduced and they can be shipped in sterile conditions.

However, the *in vitro* storage of large quantities of material induces various problems: laboratory management of plant material which needs to be regularly subcultured, risks of genetic variation, which increase with *in, vitro* storage duration, and can lead to the loss of trueness to type.

Cryopreservation

Cryopreservation is a method to conserve the plant materials at ultra low temperatures. A cryopreservation process comprises the following successive steps which have to be defined for every species: choice of material, pretreatment, freezing, storage, thawing, post-treatment.

Choice of material

As a general rule, material will be chosen as young and as meristematic as possible. Indeed, cells in this type of material are the most likely to withstand freezing. The material can be sampled from *in vivo* or *in vitro* plants. *In vitro* material is generally preferable, since the explants are already miniaturised, free of superficial contamination and may also be pathogen free. The physiological stage of the material is very important.

With carnation meristems, survival decreases progressively with their rank on the shoot axis, starting from the terminal meristem

Pretreatment

The pretreatment corresponds to a culture of the material for a certain period of time (several minutes to a few days) under conditions which prepare it to the freezing process. It is carried out using various cryoprotective substances like sucrose, sorbitol, mannitol, dimethylsulfoxide, polyethylene glycol, which differ greatly one from the other by their molecular weight and their structure.

Freezing

Different types of freezing processes can be carried out: ultra-rapid, rapid, or slow freezing. In the latter case, a programmable freezing apparatus will be needed in order to obtain precise and reproducible freezing conditions.

Storage

The maximal storage duration is theoretically unlimited, provided that the samples are permanently kept at or near the temperature of liquid nitrogen.

Thawing

In the majority of the cases, thawing is carried out rapidly by immersing the cryotubes containing the samples in a water-bath thermostated at around 40° C. The aim is to avoid fusion during thawing of the ice microcrystals formed during freezing.

Post-treatment

Post-treatment consists of culturing the material under conditions that ensure its optimal recovery in the best possible conditions. Cryoprotective substances are progressively eliminated by rinsing, dilution or diffusion, for they are toxic if kept too long in contact with the material.

Viability assessment

In many cases, regrowth is very slow. The only definitive assessment of viability is regrowth after thawing. However, it is very important to know as soon as possible if material is living after freezing. Two main tests exist in order to measure the viability of the material, which can be applied very rapidly after thawing. These tests are:

-FDA (fluorescein diacetate): FDA is absorbed by the living cells and transformed into fluorescein, whose fluorescence is induced by UV irradiation. This test is quantitative in that the percentage of fluorescing cells can be counted.

- TTC (2, 3, *5* - triphenyl tetrazolium chloride): TTC is reduced to formazan, colored red, by respiration in the mitochondria of the living cells. This test is quantitative for cell suspensions but is only qualitative for large tissues and organs.

The major disadvantage of viability tests is that they are destructive. Moreover, the FDA test is very precise for estimating viability, but gives no information on the capacity of the cells to proliferate.

Non destructive methods for estimating the viability of the material are sought, such as chromatographic analysis of volatile hydrocarbon production (ethylene, ethane) by cryopreserved tissues.

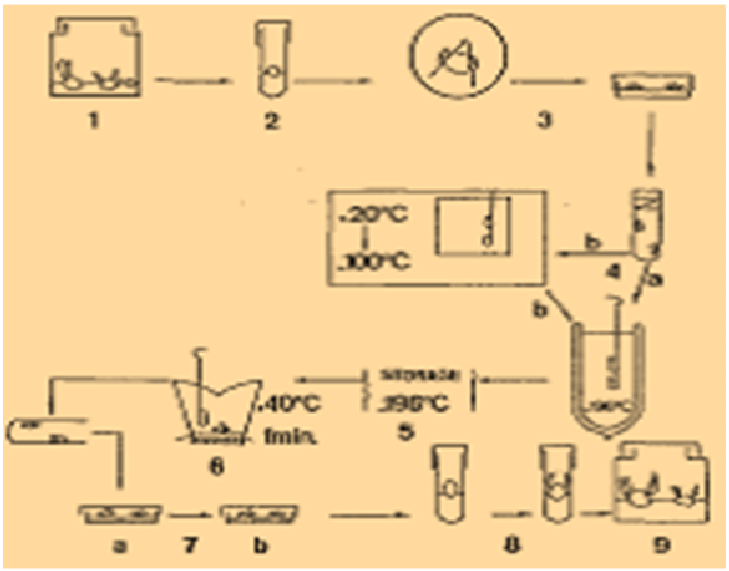


Fig.1. Schematic representation of the oil palm somatic embryo cryopreservation process.

1. Starting material.
2. Production of embryoid clumps for freezing: 2 months on sucrose 0.3 M.
3. Dissection of clumps and 7-day pretreatment on sucrose 0.75 M.
4. Freezing: a) rapid b) programmed.
5. Storage in liquid nitrogen (- 196°C).
6. Thawing: 1 min at 40° C.

7. Post-treatment:

a) 1 wk on sucrose 0.3 M + 2.4-D

b) 2 wks on sucrose O.1Mf 2,4-D 10-6M.

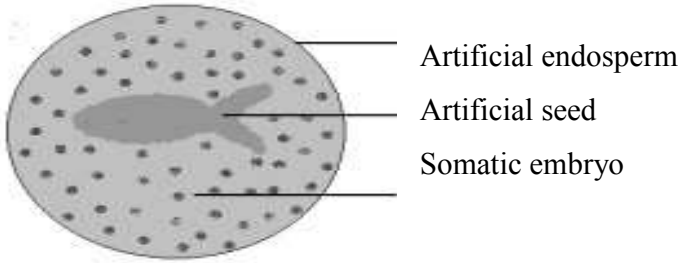
8. Successive transfers on multiplication medium: resumption

9. Transfer to jars to allow cultures to grow

Synthetic seeds

In the conventional plant tissue culture for clonal propagation, storage and transportation of propagules for transplantation is a major problem. To overcome this problem during 1980s and 1990s, the concept of synthetic or artificial seeds became popular, where somatic embryos were encapsulated in a suitable matrix (e.g. sodium alginate), along with substances like mycorrhizae, insecticides, fungicides and herbicides. In India, this technique of synthetic seeds was standardized and practiced for sandalwood and mulberry at BARC (Bombay).

Synthetic seeds have many advantages including the following: (i) they can be stored up to a year without loss of viability; (ii) they are easy to handle, and useful as units of delivery; (iii) they can be directly sown in the soil like natural seeds and do not need hardening in green house. The only limitation of synthetic seeds, is the high cost of their production. However, this may go down in future, so that the synthetic seeds will become popular at the commercial scale in due course of time.



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Questions (For Lecture # 6 to 12)

1.The process of *de novo* organ formation referred as

Organogenesis b) Morphogenesis c) Both a and b d) Rhizogenesis

Ans : Both a and b

2.Production of true-to-type plants using tissue culture protocol is termed as

Micrografting b) Macropropagation c) Macrografting d) Micrografting

Ans : Micrografting

3.Totipotency is not found in

Terminal leaf b) terminal bud c) terminally differentiated structures such as sieve tubes/tracheids d) all the above

Ans : terminally differentiated structures such as sieve tubes/tracheids

4.Adventitious *de novo* formation of roots is called as

Caulogenesis b) Rhizogenesis c) organogenesis d) all the above

Ans : Rhizogenesis

5.Embryos formed from cells or tissues of plant body i.e., other than zygotic or seed embryos are called as

a) embryoids b) somatic embryos c) both a and b d) plant embryos

Ans : both a and b

6.Embryos formed directly from the microspores are termed as

a) androgenesis b) gynogenesis c) microgenesis d) embryogenesis

Ans : androgenesis

7.Production of haploid plants from female egg nucleus or ovum is called as

a) androgenesis b) gynogenesis c) microgenesis d) embryogenesis

Ans : gynogenesis

8.An individual plant selected for propagation is referred as

a) Mother plant b) stock plant c) ortet d) all the above

Ans : all the above

9.'Undefined' part of the tissue culture medium comprises

a) tender coconut water or coconut milk b) fruit juices c) yeast extract d) all the above

Ans : all the above

10.Uptake of plant nutrients is influenced by

a) Concentration of nutrients b) pH or physiological status of tissu c) Temperature d) all

Ans : all the above

11.The possible way of uptake of nutrients by plant cells in tissue culture media is via

a) Stomata b) mesophyll c) both a and b d) none of the above

Ans : stomata

12.The buffer which is commonly used in plant tissue culture media is

a) KH_2PO_4 b) organic acids c) TRIS d) all the above

Ans : all the above

13.The first cytokinins to be discovered

a) Kinetin b) 6-furfuryl aminopurine c) both a and b d) dihydrozeatin

Ans : both a and b

14.Who proposed the concept of the "cell theory"?

a) Schleiden and Schwann b) Skoog c) Murashige d) Karry Mullis

Ans : Schleiden and Schwann

15.Name the substance added to the culture medium to induce callus induction

a) auxins b) cytokinins c) GA_3 d) Ethylene

Ans : auxins

16.Which among the following techniques does not belong to plant tissue culture

a. micropropagation b. anther culture c. replica plating d. protoplast culture

Ans : replica plating

17.Virus free plants can be produced by

a. meristem tip culture b. callus culture c. somatic embryogenesis d. suspension culture

Ans : meristem tip culture

18.The first commercially released GM crop in India is

a. Cotton expressing *cryIAb* gene b. Brinjal expressing *cry1Ab* gene c. Corn expressing *cryIAb* gene d. Cotton expressing *cryIAc* gene

Ans : Cotton expressing *cryIAb* gene

19.The term somaclonal variation was coined by

a) Murashige and Skoog b) Karp and Maddock c) Gamborg and Phillips d) Larkin and Scowcroft

Ans : Larkin and Scowcroft

20.During *in vitro* multiplication of plant, browning can be controlled by

a) Nurse culture b) Osmotic treatment of explant c) Addition of antioxidant in the medium d) Increasing agar concentration

Ans : Addition of antioxidant in the medium

21.Cybrids are obtained by

crossing a male sterile CMS line with a new line which is going to be converted into a CMS line

b. By fusing cytoplasm of CMS line with normal protoplasts of the line into which CMS is to be transferred c. Both a and b d. None of the above

Ans: By fusing cytoplasm of CMS line with normal protoplasts of the line into which CMS is to be transferred

22.Abscissic acid controls

a. Shoot elongation b. cell elongation and cell wall formation c. leaf fall and dormancy d. cell division

Ans: leaf fall and dormancy

23.Embryogenesis in plant tissue culture is induced by

a. Ethylene b. Abscissic acid c. Gibberellic acid d. Auxin

Ans: Ethylene

24.Growth factors, amino acids and vitamins are sterilized by

a. Filter sterilization b. chemical sterilization c. Steam sterilization d. air sterilization

Ans: Filter sterilization

25.An anti-malarial drug "Quinine" is a secondary metabolite produced by the plant

a. *Papaver somniferum* b. *Jasminum* spp. c. *Cinchona officinalis* d. *Vanilla* spp.

Ans: *Cinchona officinalis*

26.Haploid production through anther culture is referred as

a. Sporogenesis b. Androgenesis c. Gynogenesis d. organogenesis

Ans: Androgenesis

27.Haploids can be diploidized to produce homozygous plants by

a. Gibberellic acid b. Auxins c. Cytokinins d. Colchicine

Ans: Colchicine

28.Flavr Savr® is a genetically engineered tomato showing

a. delayed ripening b. resistance against fruit borer c. increased aroma

d. increased lycopene

Ans: delayed ripening

29.Genes present in the T-DNA of *Agrobacterium tumefaciens* are called as

a. Transfer genes b. Phytooncogenes c. Pseudogenes d. Polygenes

Ans: Phytooncogenes

30.Vitamin A rich rice will have over accumulation of

a. alpha carotene b. Beta carotene c. ascorbic acid d. Xanthoxin

Ans: beta carotene

31.Sciophytes are

a. Sun loving b. Shade loving c. winter crops d. Water loving

Ans: Shade loving

32.Cofactor of the enzyme Urease in higher plants

a. Cobalt b. Nickle c. Zinc d. Molybdenum

Ans: Nickle

33.Group of plants produced from a single plant through asexual reproduction

a. somaclone b. Tillers c. Clone d. Adventitious shoots

Ans: Clone

34.Antinutritional factor present in *Brassica* spp.,

a. Erucic acid b. Phytic acid c. Trypsin inhibitor d. Haemagglutinin

Ans: Erucic acid

35.Generation of homozygous doubled haploids from anthers of F1 hybrids is called as

a. anther culture b. chromosome doubling c. hybrid sorting d. micropropagation

Ans: . Hybrid sorting

36.Conversion of less valuable compounds into more useful compounds through cell culture is called as

a. biotransformation b. genetic engineering c. secondary metabolite d. bioconversion

Ans: biotransformation

37.Chemical used in the production of artificial seeds

a. Agarose b. calcium alginate c. gelrite d. acrylamide

Ans: Agarose

38.Organelle transfer is possible through

a. cybrid development b. particle gun bombardment c. cell fusion d. all the above

Ans: cybrid development

39.Cytoplasmic genes are called as

a. epigenes b. Plasmon c. karyons d. pseudogenes

Ans: Plasmon

40.---------- is a mass of undifferentiated cells

(a) Primordia (b) Somatic embryo c) Callus (d) SAM

Ans: Callus

41.Protoplast was discovered by ----------

(a) Mendel (b) Purkinje (c) Darwin d) Morgan

Ans: Purkinje

42.First successful transgenic fruit plant is produced in

a) Apple b) pear c) plum d) peach

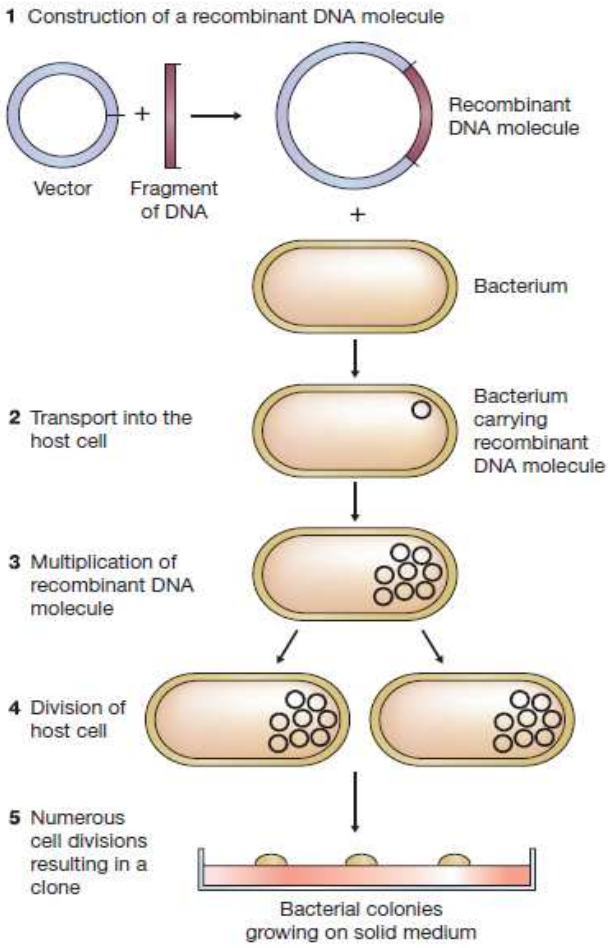
Ans: pear

Lecture.34

Genetic Engineering: Present Status and Future Trends

Genetic Engineering or Recombinant DNA technology

Recombinant DNA (rDNA) is the DNA, generated *in vitro,* by covalently joining DNA molecules from different sources. The technology associated with construction and application of recombinant DNA is referred to as genetic engineering or genetic manipulation or rDNA technology. The basis of rDNA technology is a set of key enzymes and techniques, which allow DNA to be manipulated and modified precisely.



Any rDNA experiment has the following four essential steps:

1. Generating DNA fragments,
2. Cutting and joining the DNA fragments to vector DNA molecules,
3. Introducing the vectors carrying the foreign DNA into host cells where they can replicate and
4. Selecting the clone(s) of recipient cells that have acquired the recombinant DNA molecules.

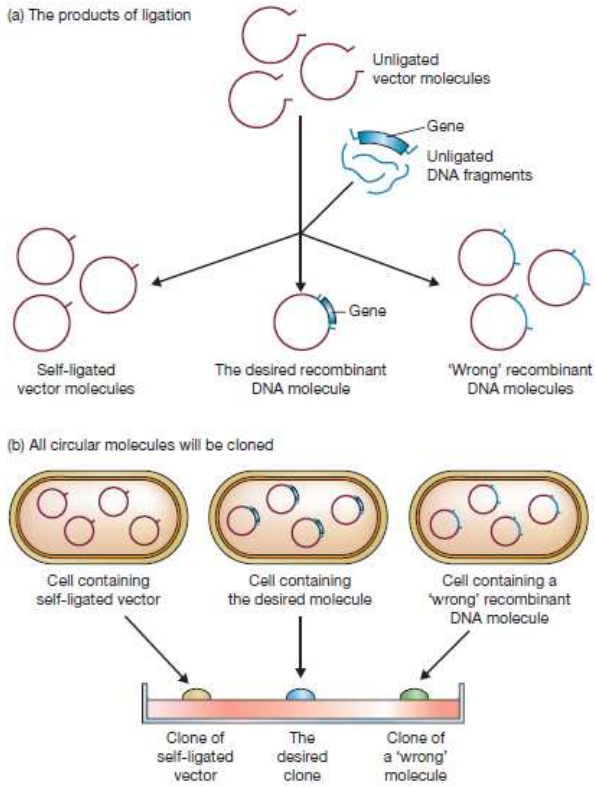
Vectors

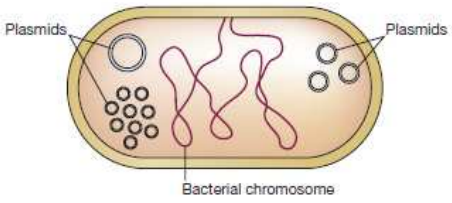
A DNA molecule needs to display several features to be able to act as a vector for gene cloning. Most importantly it must be able to replicate within the host cell, so that numerous copies of the recombinant DNA molecule can be produced and passed to the daughter cells. A cloning vector also needs to be relatively small, ideally less than 10 kb

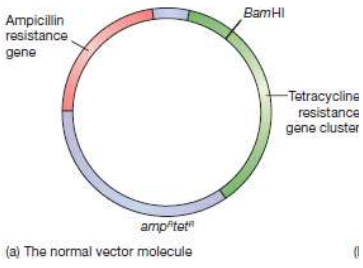
in size, as large molecules tend to break down during purification, and are also more difficult to manipulate. Two kinds of DNA molecule that satisfy these criteria can be found in bacterial cells: plasmids and bacteriophage chromosomes.

Plasmids

Plasmids are circular molecules of DNA that lead an independent existence in the bacterial cell. Plasmids almost always carry one or more genes, and often these genes are responsible for a useful characteristic displayed by the host bacterium. For example, the ability to survive in normally toxic concentrations of antibiotics such as chloramphenicol or ampicillin is often due to the presence in the bacterium of a plasmid carrying antibiotic resistance genes. In the laboratory, antibiotic resistance is often used as a **selectable marker** to ensure that bacteria in a culture contain a particular plasmid.







DNA manipulative enzymes can be grouped into four broad classes, depending on the type of reaction that they catalyze:

1. **Nucleases** are enzymes that cut, shorten, or degrade nucleic acid molecules.
2. **Ligases** join nucleic acid molecules together.
3. **Polymerases** make copies of molecules.

4. Modifying enzymes **remove or add chemical groups.**

Vector types and insert that can be inserted into the vector

Vector type	Insert size
Plasmid	< 5 Kb (but upto 20 Kbp has been tried)
Phagemid	< 5 Kb (but upto 20 Kbp has been tried)
Bacteriophage λ-insertion vector	0-10 Kbp
Bacteriophage λ- replacement vector	9-23 Kbp
Cosmid	30-45 kbp
Bacterial artificial chromosome - BAC	> 300 Kbp
Bacteriophage P1 derived artificial chromosome -PAC	~ 100 Kbp
Yeast artificial chromosome - YAC	> 2000 Kbp

Plant Transformation

Plant transformation has become widely adopted as a method to both understand how plants work and to improve crop plant characteristics. Plant transformation depends on the stable introduction of transgene(s) into the plant genome. Various methods have been developed to achieve this and many plant species have been successfully transformed. As an initial step the gene of interest is isolated and then integrated into a suitable vector. The recombinant vector is used for transformation in variety of methods. These methods were broadly grouped into two classes. 1. Direct Gene transfer 2. Indirect Gene transfer.

Direct gene Transfer

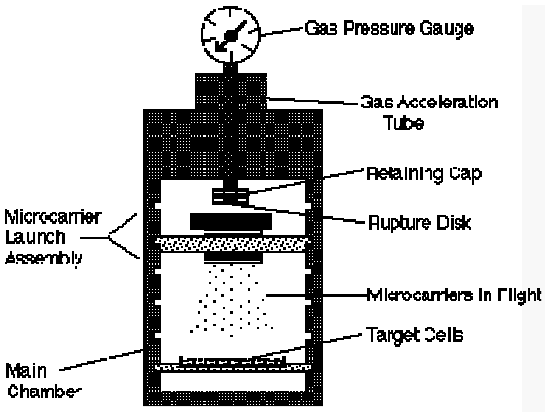
The term ‘direct gene transfer’ is used to discriminate between methods of plant transformation that rely on the use of *Agrobacterium* (indirect methods) and those that do not (direct method). Direct gene transfer methods rely on the delivery of large amounts of ‘naked’ DNA whilst the plant cell is transiently (rapidly) permeabilised. The main types of direct gene transfer methods will be considered in detail below. others, less reproducible methods are laser-mediated uptake of DNA, ultrasound and in plant exogenous application.

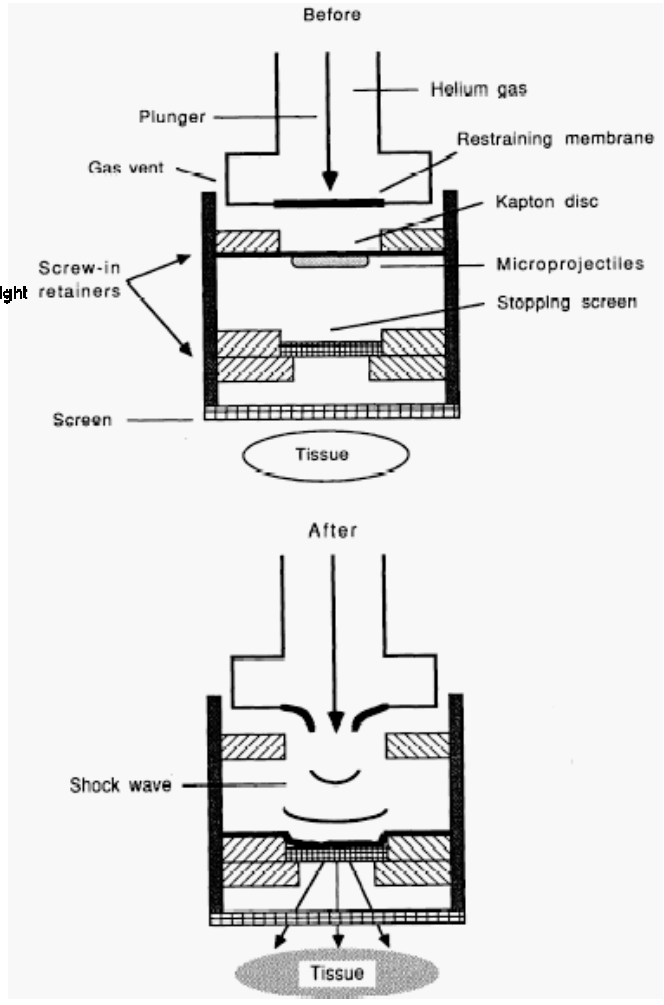
1. Particle bombardment / gene gun method/ biolistic method / micro projectile

Particle bombardment or biolistic or gene gun is the most important and effective direct gene transfer method in regular use. In this technique, tungsten or gold particles are coated with the DNA that is to be used to transform the plant tissue. The particles are propelled at high speed into the target plant material, where the DNA is released within the cell and can integrate in to the genome. The delivery of the DNA using this technology has allowed transient gene expression (which does not depend on integration of the transgene into the plant genome) to be widely studied, but integration of the transgene occurs very rarely. In order to generate transgenic plants, the plant material, the tissue culture regime and the transformation conditions have to be optimized quite carefully. Developments to this technology led to the production of a number of systems, such as an electrostatic discharge device and others based on gas flow. Of the later type, a commercially produced, helium-driven, particle bombardment apparatus (PDS-1000 He) has become the most widely used.

Plant tissues used for bombardment are generally of two types: primary explants that are bombarded and then induced to become embryogenic; or proliferating embryogenic cultures that are bombarded and then allowed to proliferate further and subsequently regenerate. This method is found to be suitable for cereals which are otherwise recalcitrant to *Agrobacterium* mediated transformation. However, the major complication is the vector DNA is often rearranged and transgene copy number can be very high. Despite the high copy number, the single locus may have benefits for subsequent breeding program.

Brief mechanism of biolistic method





The recombinant plasmids are constructed in such a way that it carries the gene of interest, selectable marker gene (like *hyg*- confers resistance to hygromycin) and reporter gene (e.g. *gusA*- which can be easily assayed by histochemically). The plasmid is coated on to gold particles (which are termed as microcarrier) for bombardment into the plant cell. Gold or tungsten particles (beads or balls) are used as microcarrier and they are prepared by mixing plasmid DNA with beads along with spermidine (a polyamine). The microcarrier are then mixed, washed with ethanol and finally resuspended in ethanol. The microcarriers are then applied to the macrocarrier membrane as an ethanol suspension and are allowed to dry on to the macrocarrier. The plant material used for transformation is embryogenic callus derived from mature seeds on MS1 medium. The embryogenic callus is arranged in a petridish containing high concentration of maltose to generate a high osmoticum prior to particle bombardment of the plant tissue.

Once the vacuum in the lower part of the apparatus is established, the helium pressure above the rupture disc is increased until at 1100 psi (or whatever pressure the rupture disc is designed to rupture at) the rupture disc bursts. This propels the fragments of the macrocarrier and the projectiles down the chamber. The macrocarrier is stopped at the stopping plate and allowing only the microcarrier to pass through and hit the plant material. Various parameters can be optimized in this system: the distance between the stopping plate and the plant material can be varied; the pressure at which the rupture disc bursts can also be selected. Varying these parameters allows the speed and pattern of the microcarriers to be adjusted to suit the needs of the plant material being transformed.

One day after bombardment, the embryogenic callus is transferred to MS1 medium. After 1 week, the embryogenic callus is transferred to selection medium (MS1 medium+hygromycin) and incubated in dark for 2 weeks. This allows only the transformed cell to proliferate. Then the surviving callus is transferred to shoot and root regeneration medium. The presence (Southern blotting) and expression of the gene interest is verified by different means (Northern blotting and Western Blotting) and the positive plants are selected. Young plantlets are acclimatized in growth chambers and can then be transferred to soil and grown to maturity.

Polyethylene glycol (PEG) mediated transformation

Plant protoplasts (plant cells without cell wall) can be transformed with naked DNA by treatment with PEG in the presence of divalent cations (usually calcium). The PEG and the divalent cations destabilize the plasma membrane of the plant protoplast and render it permeable to naked DNA. Once entered into the protoplast the DNA enters the nucleus and integrates into the genome.

Plant protoplasts are not easy to work with, and the regeneration of fertile plants from protoplasts is problematical for some species, limiting the usefulness of the technique. The DNA used for transformation is also susceptible degradation and rearrangement. Despite these limitations, the technique does have the advantages that protoplasts can be isolated and transformed in large numbers from a wide range of plant species.

Electroporation

The Electroporation of cells can be used to deliver DNA into plant cells and protoplasts. The vectors used can be simple plasmids. The genes of interest require plant regulatory sequences, but no specific sequences are required for integration. Material is incubated in a buffer solution containing DNA and subjected to high-voltage electrical pulses. The DNA migrates then through high voltage induced pores in the plasma membrane and integrates into the genome. Initially, protoplasts were used for transformation, but one of the advantages of the system is that both intact cells and tissues (such as callus cultures, immature embryos and inflorescence material) can be used. However, the plant material used for Electroporation may require specific treatments, such as pre- and post-electroporation incubations in high osmotic buffers. The efficiency of electroporation is very dependent on the condition of the plant material used and the electroporation and tissue treatment conditions chosen. The advantage of this system is all the electroporated cells are in the same physiological state. This method can be used both for plant and animal transformation.

Microinjection

In this method, which is mostly used for animal cells, DNA solution is directly injected into the nucleus of the cell. Typically, a microinjection assembly consists of a low power stereoscopic dissecting microscope and two micromanipulators, one for a glass micropipette to hold the nucleus by partial suction and the other for a glass injection needle to introduce the DNA into the nucleus. Generally, 2 picolitre (2 X 10^{-12} litre) of DNA solution is injected into the nucleus. The transgene integration occurs at random sites in the genome.

Other methods includes macroinjection, liposome mediated transformation, ultra sound mediated DNA transfer, DNA transfer via pollen etc is also used in plant transformation.

Indirect gene transfer method

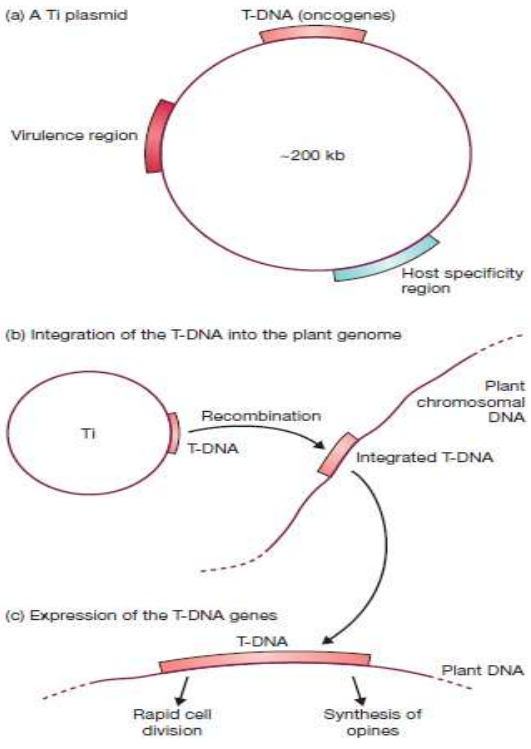
***Agrobacterium* mediated transformation**

Agrobacterium tumefaciens is a soil borne, Gram-negative bacterium, causative agent of 'crown gall' disease, an economically important disease of grapes, walnuts, apples and roses. The ability to cause grown gall (tumorous tissue growth) depends on the ability of *Agrobacterium* spp. to transfer bacterial genes into the plant genome. This is an unique example of inter-kingdom gene transfer and biotechnologists made use of this unique capability in development of plant transformation methods.

Crown gall formation depends on the presence of a plasmid in *Agrobacterium tumefaciens* known as the "Ti" (tumour inducing) plasmid. Part of this plasmid (the T (transfer) DNA region) is actually transferred from the bacterium into the plant cell, where it becomes integrated into the plant genome. The T-DNA carries genes involved in both hormone (auxin and cytokinin) biosynthesis and the biosynthesis of novel plant metabolites called opines and agropines. The production of auxin and cytokinin causes the plant cells to proliferate and so form the gall. The opines and agropines are used by *Agrobacterium.* Opines and agropines are not normally part of plant metabolism and are very stable chemicals, which provide carbon and energy source that only *Agrobacteriu*m can use.

Molecular mechanism of T-DNA transfer

Wounded plant cells release phenolic substances and sugars that are sensed by VirA protein, which activates VirG protein by phospharylation. VirG induces the expression of all the genes in the *vir* region of the Ti plasmid. Gene products of the *vir* genes are involved in variety of process. VirD1 and VirD2

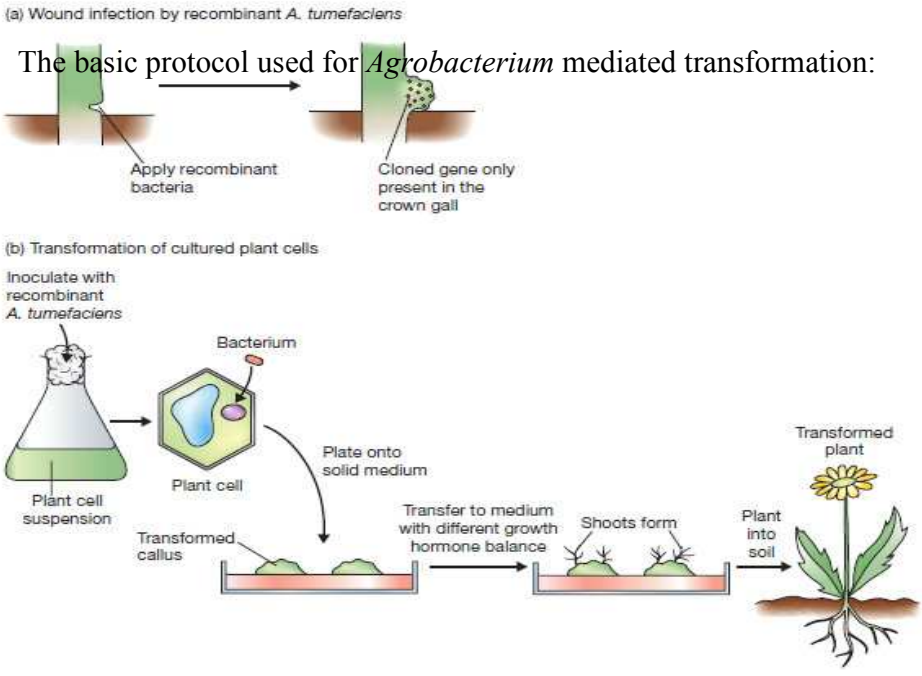


proteins are involved in single stranded T-DNA production, protection and export and VirB products forms the transfer apparatus (molecular bridge). The single stranded T-DNA (associated with VirD2) and VirE2 are exported through the transfer apparatus. In the plant cells, the T-DNA coated with VirE2 and VirD2 interacts with different plant proteins which are then attached to the T-DNA and influence transport and integration. The T-DNA/VirD2/VirE2/plant protein complex enters the nucleus through the nuclear port complex. Integration into the plant chromosome occurs through illegitimate recombination.

Agrobacterium mediated plant transformation protocol:

1. Explant: The explant used in transformation experiments must be capable of producing whole plants by regeneration and should contain high proportion of cells that are competent for transformation.
2. Vector construction: Vectors used in this system are derivatives of Ti plasmid. They are, however, extensively modified so that most of the features of a natural Ti plasmid are removed, only the left and right border sequences being used to ensure transfer of the T-DNA region between them. The vector also contains a selectable marker on the T-DNA so that transformed plants can be identified, as well as a separate selectable marker (outside the T-DNA) to enable identification of the transformed bacteria. Virulence genes required for transfer of the T-DNA either located on in this same plasmid or separate plasmid.

The basic protocol used for *Agrobacterium* mediated transformation:



1. Suitable plant tissue, to be used as a source of explants, is removed from the donor plant and sterilized. For tobacco transformation, leaves are ideal.
2. The leaf tissue is cut into small pieces (using a scalper or cork-borer) and placed into a culture of *Agrobacterium* (Which contains the recombinant vector) for about 30 minutes, a process known as co-cultivation. During this process, the bacteria attaches to the plant cells, since the medium contains Acetosyrengone – a phenolic compound (released when the plants wounded). The explants are subsequently removed from the bacterial culture, excess bacterial culture blotted off and then placed on to the solid MS medium that contains no selective agent.
3. The incubation of the explants with *Agrobacterium.* is allowed to continue for 2 days to allow transfer of the T-DNA to the plant cells. This step is called as co-cultivation.
4. The explants are removed from the medium and washed in an antibiotic solution (such as cefotaxime) that kills *Agrobacterium.*
5. The explants are transferred to fresh solid medium. This medium is supplemented with a selective agent (depend on which selectable marker gene is present in the T-DNA of the vector) to prevent the growth of non-transformed plant cells.
6. The selected transformed explants are then transferred to shoot and root regeneration medium.
7. Finally transformed plants were confirmed by PCR, southern blotting for the presence of the foreign gene and by Northern and Western Blotting for the expression of the foreign gene.

Applications of Transgenic plants

Transgenic research in crop plants offered several applications in horticulture. It includes quality improvement such as

- Oils, such as soybean and canola oils, developed to contain more stearate, making margarine and shortenings more healthful
- Peas grown to remain sweeter and produce higher crop yields
- Smaller, seedless melons for use as single servings

- Bananas and pineapples with delayed ripening qualities
- Peanuts with improved protein balance
- Fungal resistant bananas
- Tomatoes with a higher antioxidant (lycopene) content than current varieties
- Potatoes with a higher solids content (higher starch) than conventional potatoes
- Reducing the amount of oil absorbed during processing of foods like french fries or potato chips
- Fruits and vegetables fortified with or containing higher levels of vitamins such as c and e, to potentially protect against the risk of chronic diseases such as cancer and heart disease
- Rice with increased levels of provitamin a (referred as golden rice) and fe
- Garlic cloves, producing more allicin, possibly helping to lower cholesterol levels
- Higher-protein rice, using genes transferred from pea plants
- Strawberries, containing increased levels of ellagic acid, a natural-cancer fighting agent
- Peppers, strawberries, raspberries, bananas, sweet potatoes and melons that are enhanced for better nutrition and quality
- Strawberries with higher crop yields and improved freshness, flavor and texture

Besides transgenic plants have revolutionized the agriculture in several realm such as development of insect resistance (Bt-cotton, Bt-corn) and disease resistance, herbicide resistance, and grain quality improvement. For detailed discussion refer the applications of biotechnology dealt in previous lecture.

Future Trends

Molecular Pharming or Biopharming is the rapidly evolving transgenic technology and it can be simply defined as production of pharmaceutical compounds using recombinant microbes / plants / animals.

The concept of biopharming is not new. Many common medicines, such as codeine, morphine, bulk laxatives, and the anticancer drugs such as taxol and vincristine have long been purified from plants. But biopharming's great promise lies in using genetic modification i.e., techniques to make wild (nontransformed) plants to do drastic new things. Genetic modification has been applied to plants for decades in order to improve their nutritional value and agronomic traits (yield, pest- and drought-resistance, etc.). The production of high value-added substances through gene manipulation is a logical, straightforward extension.

Biopharming offers tremendous advantages over traditional methods for producing pharmaceuticals. There is great potential for reducing the costs of production. The energy for product synthesis comes from the sun, and the primary raw materials are water and carbon dioxide. And if it becomes necessary to expand production, it is much easier to plant a few additional hectares than to build a new bricks-and-mortar manufacturing facility. Another major advantage is that vaccines produced in this way will be designed to be heat-stable so that no refrigeration chain from manufacturer to patient will be required. This would have a great application in developing countries, especially in the tropics and throughout Asia and Africa.

Globally, several companies are involved in biopharming, about half have products in clinical trials, and at least one biopharmed medical diagnostic is being sold. The spectrum of products is broad, ranging from the prevention of tooth decay and the common cold to treatments for cancer and cystic fibrosis. In April 2008, California-based Ventria Bioscience (www.ventria.com) reported favorable clinical results with two human proteins biopharmed in rice and used to treat pediatric diarrhea.

Plants as bioreactors

Plants are a useful alternative to animals for recombinant-protein production because they are inexpensive to grow and scale-up from laboratory testing to commercial production is easy. Therefore, there is much interest in using plants as production systems for the synthesis of recombinant proteins and other speciality chemicals. There is some concern that therapeutic molecules produced in animal expression systems could be contaminated with small quantities of endogenous viruses or prions, a risk factor that is

absent from plants. Furthermore, plants carry out very similar post-translational modification reactions to animal cells, with only minor differences in glycosylation patterns. Thus plants are quite suitable for the production of recombinant human proteins for therapeutic use. A selection of therapeutic proteins that have been expressed in plants is listed in Table.

The first such report was the expression of human growth hormone, as a fusion with the *Agrobacterium* Nopaline synthase enzyme, in transgenic tobacco and sunflower. Tobacco has been the most frequently used host for recombinant-protein expression although edible crops, such as rice, are now becoming popular, since recombinant proteins produced in such crops could in principle be administered orally without purification. The expression of human antibodies in plants has particular relevance in this context, because the consumption of plant material containing recombinant antibodies could provide passive immunity (i.e. immunity brought about without stimulating the host immune system).

Antibody production in plants was first demonstrated by Hiatt and During team, who expressed full-size immunoglobulins in tobacco leaves. Since then, many different types of antibody have been expressed in plants, predominantly tobacco, including full-size immunoglobulins, Fab fragments and single-chain Fv fragments (scFvs). For example, a fully humanized antibody against herpes simplex virus-2 (HSV-2) has been expressed in soybean. Even secretory IgA (sIgA) antibodies, which have four separate polypeptide components, have been successfully expressed in plants. This experiment involved the generation of four separate transgenic tobacco lines, each expressing a single component, and the sequential crossing of these lines to generate plants in which all four transgenes were stacked. Plants producing recombinant sIgA against the oral pathogen *Streptococcus mutans* have been generated, and these plant-derived antibodies (‘plantibodies’) have recently been commercially produced as the drug CaroRxTM, marketed by Planet Biotechnology Inc.

Table. Selective examples of recombinant human therapeutic proteins expressed in plants

Species	Recombinant human product
Tobacco, sunflower (plants)	Growth hormone
Tobacco, potato (plants)	Serum albumin
Tobacco (plants)	Epidermal growth factor
Rice (plants)	α-Interferon
Tobacco (cell culture)	Erythropoietin
Tobacco (plants)	Haemoglobin
Tobacco (cell culture)	Interleukins-2 and 4
Tobacco (root culture)	Placental alkaline phosphatase
Rice (cell culture)	α_1-Antitrypsin
Tobacco (seeds)	Growth hormone
Tobacco (chloroplasts)	Growth hormone

Questions

1. Molecular cloning is an _____ technique.

a) *In vivo* **b)** *in vitro* **c)** both a and b **d)** none of the above

Ans: both a and b

2. The restriction enzyme which is used widely in molecular cloning experiments are

a) Type I **b)** type II **c)** type III **d)** all the above

Ans: type II

3. Source of DNA for cloning experiments is

a) Genomic DNA **b)** cDNA **c)** PCR product or previously isolated clone or chemically synthesized oligonucleotides **d)** all the above

Ans: all the above

4. Random cleavage of DNA can be achieved by

a) Mechanical shearing (sonication, vortexing) **b)** chemical treatment (acid/alkali hydrolysis) **c)** non-specific endonuclease **d)** all the above

Ans: all the above

5. Precise, sequence specific cutting of DNA by restriction enzymes usually produce

a) Blunt end **b)** sticky end **c)** both a and b **d)** none of the above

Ans: both a and b

6. The 5' or 3' end of DNA can be modified by

a) end filling and trimming with T4 DNA polymerase **b)** partial end filling with Klenow DNA polymerase **c)** addition of adaptors/linkers followed by restriction enzyme digest **d)** all the above

Ans: all the above

7. Double stranded oligonucleotides which contain restriction site used in DNA end modification are

a)Linkers **b**)adoptors (predigested linkers with restriction enzymes)
c) oligonucleotides **d)** primers

Ans: linkers

8. Predicting the orientation of insert DNA in the vector is called

a)Directional cloning(eg. use of pair of restriction enzymes) **b)** bidirectional cloning
c) both a and b **d)** none of the above

Ans: directional cloning (eg. use of pair of restriction enzymes)

9. Addition of nucleotides to the 3' end of Blunt or ragged donor DNA by terminal dexoynucleotidyl transferase is termed as

a) homopolymer tailing **b)** poly-A insertion **c)** both a and b **d)** none of the above

Ans: homopolymer tailing

10. An ideal cloning vector displays which one of the following property?

a) episomal (not integrated into host genome) **b)** replicate autonomously **c)** allow selection of recombinant from non-recombinants **d)** all the above

Ans: all the above

11. An important initial step in development of naturally occurring plasmids and Bacteriophages as cloning vector is

a) construction of multiple cloning site **b)** retaining selectable or visible marker gene **c)** removal of unwanted DNA **d)** all the above

Ans: all the above

12. Usually the plasmids are developed with

a) *Col E1* replicon **b)** Bacteriophage M13 origin of replication **c)** both a and b **d)** none of the above

Ans: *Col E1* replicon

13. Selection of recombinant DNA can be made easy by using

a) Insertion vectors **b)** replacement vectors **c)** both a and b **d)** none of the above

Ans: both a and b

14. Assembly of recombinant vector into Bacteriophage λ heads is called as

a) *In vitro* packaging **b)** wrapping up **c)** both a and b **d)** none of the above

Ans: *in vitro* packaging

15. Usually the scorable or selectable markers used in vectors are

a) Dominant **b)** recessive **c)** epistatic expression **d)** all the above

Ans: dominant

16. Blue white selection strategy used to select recombinant cells is based on

a) β-galactosidase **b)** β-glucosidase **c)** pepetidogalactosidase **d)** galactose

Ans: β-galactosidase

17. Recombinant λ insertion vectors are usually selected by disruption of *cI* gene which generates ____ plaques

a) Clear **b)** turbid **c)** both a and b **d)** none of the above

Ans: clear

18.λ and cosmid vectors form infection particles if the recombinant genome is ___ % of the wild type genome size

a) 100 **b)** 75-100 **c)** 105 **d)** 75

Ans: 75-100

19. Chromosome specific genomic library can be generated by chromosome separation by

a) Fluorescence activated chromosome sorting (FACS) **b)** monochromosomal somatic cell hybrids **c)** chromosome microdissection **d)** all the above
Ans: all the above

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Primrose, S. B and Twyman, R. M. 2006. Principles of gene manipulation and genomics. (Seventh Edition) Blackwell Publishing.

Slater, A., Nigel, S and M. Fowler. 2003. Plant Biotechnology: The Genetic Manipulation of Plants. Oxford Publications.

Christou and Klee. 2004. Handbook of Plant Biotechnology. Paul Christou and. Harry Klee (eds). Volumes I and II. John Wiley & Sons, UK.

Web resources

1. http://www.mcb.uct.ac.za/manual/MolBiolManual.htm
2. http://www.web-books.com/MoBio/
3. http://cls.casa.colostate.edu/TransgenicCrops/animation.html

Exercise.1
Qualitative reactions of carbohydrates

Ex. No. 1

Date: ……..

Experiment	Observation	Inference
1. Molisch's Test Add two drops of Molisch's reagent (5% napthol in alcohol) to about 2 ml of given carbohydrate solution and mix well. Incline the tube and add about 1 ml of concentrated sulphuric acid along the sides of the tube. Observe the colour at the junction of the two liquids. **2. Iodine Test** Add a few drops of iodine solution to about 1 ml of the given test solution.		

3. Fehling's Test a) To 1 ml of Fehling's Solution A, add 1 ml of Fehling's Solution B and a few drops of the test solution containing either glucose, fructose or maltose. Boil for few minutes. b) Perform the test with sucrose solution **4. Benedict's Test** a) To 2 ml of Benedict's reagent, add five drops of the test solution containing any one of the reducing sugars. Boil for 5 minutes in a water bath. Cool the solution.		

b) Perform the test with sucrose solution. **5. Barfoed's Test** To 1 ml of the test solution add about 2 ml of Barfoed's reagent. Boil it for one minute and allow to stand for a few minutes. **6. Seliwanoff's Test** To 2 ml of Seliwanoff's Reagent (Resorcinol in HCl) add two drops of the test solution and heat the mixture to just boiling. **7. Bial's Test** To 5 ml of Bial's		

Reagent (Orcinol in HCl) add 2-3 ml of sugar solution and warm it gently. When bubbles rise to the surface, cool under the tap. **8. Test for sucrose** a) Do Benedict's test with the given sucrose solution b) Add 5 drops of concentrated HCl to 5 ml of sucrose solution in another test tube. Heat for 5 min in a boiling water bath. Add 10% sodium hydroxide solution to give a slightly alkaline solution (test with litmus paper). Now perform Benedict's Test with this hydrolysed sucrose solution.		

THINGS TO LEARN

1. Mark (+) sign wherever you get positive reactions and (-) sign wherever the reaction is negative.

S. No.	***Carbohydrate solution***	***Molisch's test***	***Iodine test***	***Fehling's test***	***Benedict's test***	***Bial's test***	***Selivanoff's test***	***Osazone***
1.	*Glucose*							
2.	*Fructose*							
3.	*Xylose*							
4.	*Sucrose* *a. Before hydrolysis* *b. After hydrolysis*							
5.	*Maltose / Lactose*							
6.	*Starch*							
7.	*Gelatos*							

2. Why is sucrose not able to reduce Benedict's reagent before hydrolysis?

3. Did you get deep red colour when you performed Selivanoff's test with sucrose solution? Explain the theory behind it?

Exercise.2

Estimation of Total Sugars

Ex. No. 2

Date: ……..

Total sugars include both reducing and non-reducing sugars. There is no separate method available for quantifying non-reducing sugars, whereas total sugars are estimated by simple methods. When the amount of reducing sugars is deducted from the total sugars, the non-reducing sugar concentration is obtained.

Extraction

Total sugars are extracted from the powdered dry material using 70% (v/v) aqueous ethyl alcohol (10 ml/g.). The fresh materials are also extracted using aqueous ethyl alcohol using the same solvent in a pestle and mortar.

Reagents

- Anthrone reagent. Dissolve 200 mg anthrone in 100 ml ice-cold 95% sulphuric acid. Prepare fresh before use.
- Glucose standard: As given in the estimation of reducing sugars.

Procedure

1. Pipette out 0.2 to 1.0 ml of the extract and keep it water bath to completely evaporate the solvent:
2. Add 1 ml water to dissolve the sugars.
3. Then add 4ml anthrone reagent from a burette.
4. Heat for eight minutes in a boiling water bath.
5. Remove the tubes, cool rapidly and read the absorbance of the green colored solution at 630 nm.
6. Prepare a standard graph using 0-100 µg glucose.

Calculation

Using the standard graph calculate the amount of carbohydrate present in sample.

Result

The total sugar content of the given sample is

Things to learn

1. *What are the other method by which you can determine total sugars?*

2. *Why anthrone reagent is prepared in ice cold conditions?*

Exercise.3

Estimation of reducing sugars

Ex. No. 3

Date: ……..

All monosaccharides and many disaccharides act as reducing agents in presence of dilute alkali. They exhibit the reducing property due to the presence of free or potentially free aldehyde or keto group. The keto group or aldehyde group under alkaline conditions reduce metal ions like Cu^{++} and Ag^{+} to Cu^{+} and Ag. During this process, reducing sugars get oxidised to lower acids. Therefore quantitative estimation of reducing sugars becomes possible with any one of the reduction processes.

Aim

To determine the amount of reducing sugar present in the given unknown solution.

Principle

Glucose is oxidised to gluconic acid by iodine under alkaline conditions.

$$C_6H_{12}O_6 + 3NaOH + I_2 \longrightarrow C_5H_{11}O_5COONa + 2\,NaI + 2\,H_2O$$

A known amount of sugar is allowed to react with an excess of iodine solution and the excess iodine is back titrated against standard sodium thiosulphate solution.

- **Reagents**
- 0.1N Iodine
- 0.1N Sodium Thiosulphate
- 1N Sodium Hydroxide
- 10 N HCl (Concentrated HCl)
- 1% Starch
- Standard glucose solution (1 mg/ml)

Procedure

Pipette out 10ml of the working standard solution containing 10 mg glucose into an iodine flask. Add 10 ml of 0.1N iodine solution followed by careful slow addition of 10 ml of 0.1N NaOH. The alkali should be added drop by drop during a period of four minutes. During the addition of NaOH, red colour of iodine changes to yellow and then to colourless solution. Rinse the sides of the flask with distilled water and add about 50 ml of distilled water. Stopper the flask and leave it for 15 minutes at room temperature.

Acidify the solution in the flask with 3 ml of 10N HCl, when the red colour of iodine is regenerated. Titrate the excess free iodine against standard thiosulphate till red colour of the solution in the iodine flask changes to straw yellow. Then add a few drops of starch solution to the flask when a blue colour is formed. Continue the addition of sodium thiosulphate drop by drop till the blue colour just disappears. Note the burette reading. This is the titre value (V_1 ml).

Make up the given unknown solution to a known volume (50 ml). Find out the amount of reducing sugar present in the given unknown solution by titrating following the procedure as above (V_2 ml). For blank, pipette out 10 ml of water and titrate after adding all the reagents mentioned above (V_3 ml). Calculate the amount of reducing sugar present in 50 ml of the unknown solution as given below.

Calculation

Amount of 0.1 N thiosulphate = 0.1 N iodine consumed by 10 ml (10 mg glucose)	}	V_3-V_1 ml = x ml
Amount of 0.1N thiosulphate = 0.1 N iodine consumed by glucose in 10 ml of unknown solution	}	$V_3 - V_2$ ml = y ml
Amount of glucose in 10 ml of unknown solution	}	(10 mg / xml) X y ml = z mg

Amount of glucose in 50 ml of unknown solution	}	$\frac{(z \times 50)}{10}$ mg

Result

50 ml of the given unknown solution contains ..... mg reducing sugar.

Things to learn

1. *Name a few reducing sugars and their sources. Write down their structures.*
2. *Can we estimate the non-reducing sugars by this method? If yes, can you suggest the modifications required in this procedure?*
3. *How does the starch function as an indicator in this titration?*

Exercise.4

Estimation of amylose

Ex. No. 4

Date: ……..

Aim

Starch is composed of amylose and amylopectin. Amylose has a molecular weight of 1, 50,000 to 10,00,000 depending on its biological origin. It is now recognized that it has some elements of non-linearity. The ratio of amylose and amylopectin plays an important role in the cooking characteristics of cereal grains, especially rice.

Principle

Amylose forms a helical coil like structure in solution in which 5 to 6 glucose residues are present in each coil. Iodine forms an absorption complex with amylose to yield a blue colour. The absorbance of the blue colour is measured at 600 nm.

Reagents

- Sodium hydroxide 1 N
- Phenolphthalein indicator
- HCl, 0.1 N
- Iodine reagent: Dissolve 1 g iodine in 10 g KI in water and then make up to 500 ml.
- Standard amylose: Dissolve 50 mg amylose in 50 ml water.

Procedure

1. Weigh 50 mg rice flour, transfer to a boiling tube, add few drops of alcohol followed by 5 ml of 1 N NaOH.
2. Boil for 15 minutes in a water bath.
3. Cool and transfer the contents to a 50 ml standard flask and make up the volume with water.

4. Pipette out 2.5 ml (0.5) of the extract into a 50 ml standard flask; add little (2.0 ml) water followed by 2 drops of phenolphthalein. Add with shaking 0.1 N HCl till the pink colour **just disappears**.
5. Add 1 ml of the iodine reagent and make up to 10 ml with water.
6. Measure the absorbance at 600 nm.
7. Prepare a standard graph with 100 – 500 µg of amylose.
8. From the standard graph, determine the amount of amylose present in the sample.

Result

The amount of amylose present in the given sample = _____.

Things to learn

1. *Distinguish amylose from amylopectin*

2. *How will you prepared 1N NaOH?*

3. *What happens to starch during the process of digestion?*

Exercise.5

Colour reactions of proteins

Ex. No. 5

Date : ……

Aim

To test verify the colour reactions of protein and to know the theory behind the reactions.

Experiment	Observation	Remarks
I Precipitation reactions **1. Heat Coagulation test** Heat 2 ml of protein solution in a test tube. **2. Heller's test** Take 2 ml of concentrated HNO_3 in a test tube. Add equal volume of protein solution along the sides of the tube. Do not mix the two solutions. **3. Precipitation by Heavy Metals** Take 2 ml of the protein solution in a test tube. Add lead acetate solution until precipitation occurs. **4. Precipitation by Organic Solvents** Add 95% alcohol or acetate drop by drop to 1 ml of protein solution.		

Experiment	Observation	Remarks
5. Precipitation by Salts To a few ml of a protein solution add saturated ammonium sulphate solution drop by drop. **II. Colour reactions** **6. Xanthoproteic reaction** To about 1ml of protein solution add equal volume of concentrated nitric acid. Cool and observe the colour change. **7. Glyoxylic reaction for Tryptophan** Add 2ml of glacial acetic acid to 2 ml of the test solution. The pour about 2 ml of conc. H_2SO_4 carefully down the sides of the test tube. Observe the colour change at the junction of the two liquids. **8. Biuret Test** Add five drops of copper sulphate solution to 2ml of the test solution followed by 2ml of NaOH. Mix thoroughly and note the colour produced.		

Things to learn

1. *You would have noticed that when you handle nitric acid that part of your fingers that is in direct contact with nitric acid turn yellow. Can you explain the reaction behind it?*

2. *When hen's egg is boiled, it get solidified.*

    a. *Can you explain this phenomenon with your knowledge on proteins?*
    b. *Is it possible to rescramble the egg and get the original form? If not why?*

3. *What is a principle behind artificial curling of hair?*

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Exercise.6

Estimation of protein by biuret method

Ex. No. 6

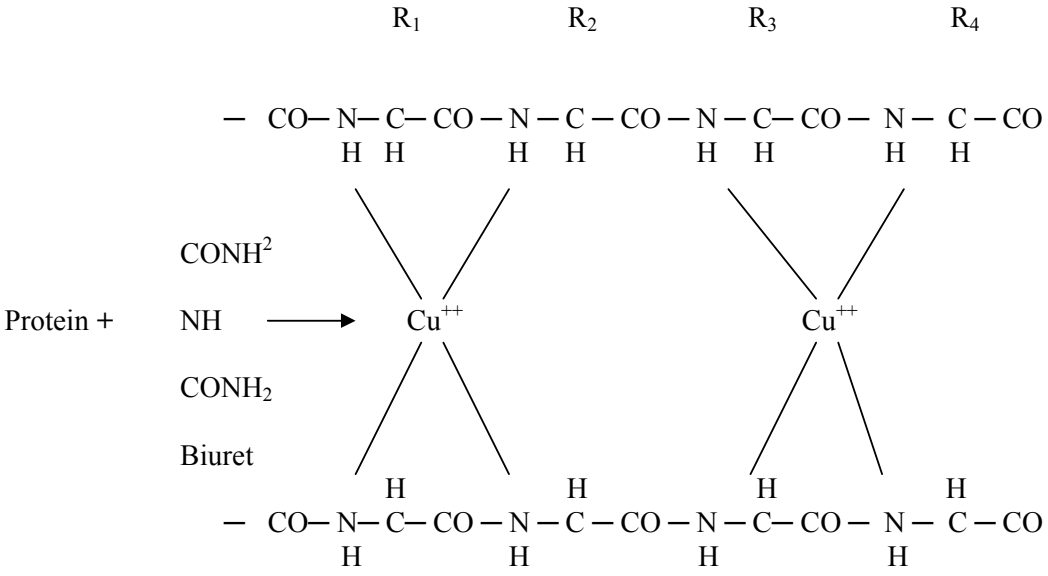
Date: ……

Proteins contain amino acids linked by peptide bonds. Differences in the number of amino acids and the sequence of amino acids result in different types of proteins. There are more than three methods for the estimation of proteins. A colorimetric method is described here.

Principle

Compounds containing two or more peptide bonds (eg. Proteins) take up a characteristics purple colour when treated with dilute copper sulphate in alkaline solution.

The colour is apparently caused by the formation of complex of the copper atom with four nitrogen atoms, two from each of two peptide chains. It requires 1-20 mg protein for colour formation.



R_1 R_2 R_3 R_4

N-atoms of the peptide bonds forming coordinate

Reagents

- 10 mg/ml protein standard
- Biuret Reagent

Procedure

Pipette out 0.2, 0.4, 0.6, 0.8 and 1.0ml of the standard protein solution into a series of tubes. Make up the given unknown solution to 10ml with distilled water and pipette out 0.2 and 0.4ml of the made up unknown solution into two more tubes. Make up the volume to 1.0ml in all the seven test tubes, by adding requisite amount of distilled water. In an eighth test tube add 1ml of distilled water to serve as blank. Add 4ml of Biuret reagent to each tube and mix thoroughly. After 30 minutes at room temperature read the absorbance of the solution at 550 nm against the reagent blank. Draw the standard graph and from the graph calculate the amount of protein present in 10ml of the given unknown solution.

Result

Ten ml of the given unknown solution contains …….. mg protein.

Things to learn

1. *What is meant by a peptide bond?*
2. *What are the uses of estimating protein content?*
3. *Name the major proteins of maize, rice wheat and milk?*
4. *Name few source rich in S-amino acids*

Exercise.7

Sorenson's formal titration of amino acids

Ex. No. 7

Date: ……..

Amino acids are the basic building blocks of proteins. They carry both amino- and carboxyl groups on the same molecule. In nature, amino acids mostly occur as proteins as well as free amino acids in many tissues. Estimation of free amino acids is so important in agricultural biochemistry and nutrition information.

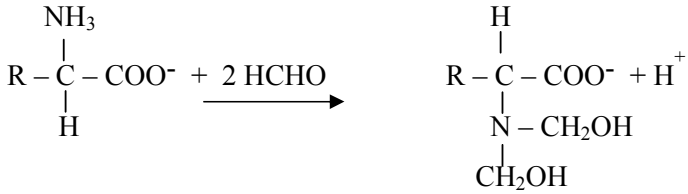
Aim

To estimate the amount of amino acid present in the whole of the given solution by Sorenson's method.

Principle

A solution containing amino acid is neutral in reaction. The carboxyl group of amino acids cannot be accurately titrated in aqueous medium with alkali because the carboxyl group and the basic amino group of the amino acid react to form zwitter ions, which are not dissociated completely at the end point of alkaline indicators (Phenolphthalein).

In the presence of formaldehyde, amino acids form the dimethylol derivatives.



Zwitter ion Dimethylol Amino acid

Hence, the presence of formaldehyde protects the basic amino group from forming zwitter ions and permits the carboxyl group to exert maximum acidity. The acidity may be titrated against standard NaOH, using the phenolphthalein as indicator.

This reaction forms the basis for Sorensen's formal titration method of estimating amino acids.

Reagents

- Formaldehyde
- 0.02 N Oxalic acid
- Approximate 0.02 N NaOH
- 0.1% Phenolphthalein

Procedure

Pipette out 10 ml of standard oxalic acid solution into a conical flask; add a few drops of phenolphthalein and titrate against sodium hydroxide. End point is the appearance of pale pink colour.

Repeat the titration for concordant values. Calculate the normality of sodium hydroxide solution using the formula

$$V_{\text{oxalic acid}} \times N_{\text{oxalic acid}} = V_{NaOH} \times N_{NaOH}$$

Make up the given unknown solution to the mark with distilled water in a 50 ml standard flask and pipette out 10 ml of the solution into a conical flask. Add 5 ml of formaldehyde and a few drops of indicator. Titrate against the standard sodium hydroxide solution (xml). Repeat for concordant values. For blank, take 5.0 ml of formaldehyde and 10 ml of water and titrate against the alkali after adding the indicator (y ml). Repeat for concordant values.

Calculation

The difference in the titre values (x-y ml) gives the amount of alkali required for neutralizing the amino acids present. Calculate the strength of amino acid using the formula,

$$V_1N_1 = V_2N_2$$

where,

V_1 = 10 ml (volume of amino acid solution); V_2 = (x-y) ml (volume of NaOH)

N_1 = Normality of amino acid solution; N_2 = Strength of NaOH

Find out the concentration of amino acid by the formula,

Strength X Equivalent weight = weight per litre (g/l)

Equivalent weight of the simplest amino acid (glycine) is 75.

Result

The strength of amino acid solution..... N

The concentration of amino acid is ..... g in 50 ml of the solution.

Things to learn

1. *What is the function of formaldehyde in Sorensen's formal titration?*

2. *Name the amino acids, which are deficient in rice proteins.*

3. *Is there any amino acid with more than one amino group / carboxyl group? If so, name them.*

4. *Name the amino acid that is deficient in puls protein.*

Exercise.8

Estimation of free fatty acids of an oil

Ex. No. 8

Date: ……..

Oils and fats are triglycerides *i.e.,* esters of fatty acids with glycerol. A little amount of free fatty acid is also present in oils along with the triglycerides. The free fatty acid content is also known as acid number / acid value of an oil. It increases during storage. The acid value of an oil or fat is defined as the number of mg of potassium hydroxide required to neutralize the free fatty acids present in one gram of oil or fat.

Aim

To estimate the free fatty acid content of the given oil.

Principle

The free fatty acid in oil is estimated by calculating the amount of sodium hydroxide required to neutralize the free fatty acid present in the oil and expressed as percent oleic acid.

Reagents

- 0.1N Sodium hydroxide
- 0.1N Oxalic Acid
- 0.1% Phenolphthalein
- Oil
- 95% Neutralized alcohol

Procedure

Pipette out 5 ml oil into a clean dry conical flask. Add 25 ml neutralized alcohol using a burette. Add a few drops of phenolphthalein and heat the contents to boiling. Titrate while hot against standard sodium hydroxide solution (x ml).

For standardization of sodium hydroxide, pipette out 10 ml of 0.1N oxalic acid into a clean conical flask, add a few drops of indicator and titrate against sodium hydroxide solution. Repeat the titration for concordant value (V_1 ml). Calculate the strength of NaOH. Find out the weight of 5 ml of oil and calculate the free fatty acid content in 100 g oil.

Calculation

I. Strength of sodium hydroxide

$$V_{NaOH} \times N_{NaOH} = V_{\text{oxalic acid}} \times N_{\text{oxalic acid}}$$

$$N_{NaOH} = (10 \times 0.1) / V_{NaOH}$$

II. Free fatty acid content

$$\text{F.F.A content} = \frac{\text{x ml} \times N_{NaOH}}{0.1} \times \frac{0.0282}{\text{weight of oil}} \times 100 \text{ g}$$

Where x ml = Volume of sodium hydroxide required to neutralize 5 ml (find out the weight of 5 ml of oil).

Result

The Free fatty acid content of the given oil is .....% Oleic acid.

Things to learn

1. *You are given (i) fresh and (ii) rancid oil. Which one of these two will have higher free fatty acid contents? Why?*
2. *Name few sources rich in saturated patty acids*

Exercise.9

Determination of iodine value of an oil or fat

Ex. No. 9

Date:……..

The iodine value is a measure of the degree of unsaturation in oil. It is constant for particular oil or fat. Iodine value is a useful parameter in studying oxidative rancidity of oil since higher the unsaturation higher is the possibility of the oil to go rancid.

Principle

Oils contain both saturated and unsaturated fatty acids. Iodine gets incorporated into the fatty acid chain wherever the double bonds exist. Hence the measure of iodine absorbed by oil gives the degree of unsaturation. Iodine value or Iodine number is defined as the (g) of iodine absorbed by 100 g of the oil.

Excess amount of Hanus iodine solution (IBr) is allowed to react with the oil for a definite period of time. IBr adds to the double bonds present in the unsaturated fatty acids. To the remaining IBr, potassium iodide solution is added and the liberated iodine is titrated against the standard sodium thiosulphate.

Reagents

- Hanus iodine reagent
- Oil
- 15% potassium iodide solution
- 1N sodium thiosulphate
- 1% freshly prepared starch solution

Procedure

Weigh about 0.25 g oil into an iodine flask and dissolve in 5 ml of chloroform. Add 20 ml of Hanus iodine reagent using a pipette, draining it in a definite time. Mix

well and allow standing in dark for exactly 30 min, with occasional shaking. Add 10 ml of 15 per cent KI solution shake thoroughly and add 10 ml of freshly boiled and cooled water, washing down any free iodine on the stopper. Titrate against sodium thiosulphate until yellow solution turns almost colourless. Add a few drops of starch as indicator and titrate until the blue colour disappears. Towards the end of the titration, stopper the flask and shake vigorously so that any iodine remaining in solution in chloroform is taken up by potassium iodide solution. Simultaneously run a blank without the sample.

Calculation

The quantity of thiosulphate required for blank minus the quantity required for the sample gives thiosulphate equivalent of iodine absorbed by the fat or oil taken for analysis.

$$\text{Iodine Number} = \frac{(B - S) \times N \times 12.60}{\text{Wt. of sample (g)}}$$

where,

B = ml thiosulphate for blank

S = ml thiosulphate for sample

N = Normality of thiosulphate

Amount of fat / oil taken should be adjusted such that the excess iodine in the added 20 ml of Hanus iodide solution has 60 percent of excess iodine of the amount added. i.e. if (B-S) is greater than B/2, repeat with smaller amount of the sample.

Result

The iodine number of the given oil = _____.

Things to learn

1. *Give the iodine value of coconut oil, groundnut oil and sunflower oil. Based on the iodine values, give your inference*.

Exercise.10

Estimation of ascorbic acid

Ex. No. 10

Date: ……..

Ascorbic acid, commonly known as vitamin C, is abundant in fresh fruits and vegetables. It is water-soluble and heat labile vitamin. Ascorbic acid is synthesized from glucose in plants.

Aim

To estimate the amount of ascorbic acid by the dye method in the given solution and also in lime fruit.

Principle

Ascorbic acid reduces the dye, 2,6 dichlorophenol indophenol (a blue coloured compound which attains red colour in acid solution) to a colourless compound. The ascorbic acid gets oxidized to dehydro ascorbic acid.

Reagents

- 4% oxalic acid
- Standard ascorbic acid solution in 4% oxalic acid (0.1 mg /ml)
- Dye solution: 42 mg sodium bicarbonate and 52 mg of dye (2,6 dichlorophenol indophenol) in 200 ml distilled water.

Procedure

Pipette out 10 ml of the working standard solution into a conical flask and add 10 ml of 4% oxalic acid and titrate against the dye (V_1 ml). End point is the appearance of pink colour, which persists for a few minutes. Repeat the titration for concordant values. The amount of the dye consumed is equivalent to the amount of ascorbic acid present.

Make up the given unknown solution to 50 ml with 4% oxalic acid. Pipette out 10 ml of this made up solution into a conical flask; add 10 ml of 4% oxalic acid and follow the titration as above (V_2 ml). Repeat the titration till you get concordant values.

Pipette out 10 ml of the diluted lime-juice and follow the titration as above (V_3 ml). Repeat the titration till you get concordant values. From the titre values, calculate the amount of ascorbic acid in

i) 50 ml of the given unknown solution and in

ii) 100 ml of fresh lime - juice.

Calculation

I. Amount of ascorbic acid present in 10 ml of working standard = 1.0 mg

Amount of dye solution required to oxidise 1.0 mg ascorbic acid = V_1 ml

Amount of ascorbic acid required to reduce V_1 ml of the dye solution = 1.0 mg.

Therefore,

Amount of ascorbic acid required to reduce

1ml of dye solution (dye factor) $= (1.0/V_1) = x$ mg

II. Amount of Ascorbic acid required to reduce 1 ml of dye solution = x mg ascorbic acid

Therefore,

Amount of ascorbic acid required to reduce

V_2 ml of dye solution $= (x \times V_2)$ mg = Y mg ascorbic acid

10 ml of the unknown solution contains = Y mg ascorbic acid

Therefore,

50 ml of unknown solution contains = Y x 50/10 mg ascorbic acid

Result

(i) The ascorbic acid content of 50 ml of the given unknown solution is ..... mg

(ii) The ascorbic acid content of 100 ml of fresh lime juice is ..... mg

Things to learn

1. *Find out the Vitamin 'C' content in tomato, Indian gooseberry and grapes.*
2. *Vitamin 'C' is not synthesized in our body, why?*

Exercise.11

Separation of amino acids by paper chromatography

Ex. No. 11

Date : ……..

Principle

Chromatography is a collective name for all separation processes where the separation of the components is effected by their partition between a stationary phase with a large surface and a moving phase, which flows over the first. In paper chromatography filter paper which is composed almost entirely of cellulose fibres, act as the inert carrier for the stationary liquid phase, usually water, while the solvent mobile phase is drawn through the paper by capillary action.

Reagents

- Chromatographic paper
- Spotting lamp / air drier
- Capillary tubes / lambda pipettes
- Standard amino acids 2 to 3 mg / ml
- Solvent
- n = Butanol : acetic acid : water 4:1:1 v/v or phenol : water 4 : 1 v/v
- Chromatographic chamber/ cabinet
- Chromogenic reagent: 0.5% ninhydrin in 95% alcohol or methyl cellosolve

Sample preparation

For free amino acids fresh biological samples are weighed and extracted with 70% alcohol. The extract may be concentrated in a rotavapor if necessary. A 10% homogenate will be sufficient for most biological samples.

Proteins may be hydrolysed in 2.5 N hydrochloric acid for the determination of amino acids. The samples containing about 10 mg proteins may be taken and hydrolysed with 5 ml 0.5N HCl under vacuum at 110°C for 18 hours. HCl may be evoporated in a rotavapor completely and the residue is taken in 70% alcohol.

Spotting

Cut Whatman filter paper No.1 or any other chromatographic paper to the required size. Draw a pencil line about 4 cm from one edge of the paper and mark points at an interval of 4 cm. Apply the unknown sample at the central spot and the standard amino acids on both sides. About 50 µl will be ideal for spotting. Spotting lamp or air drier may be used for rapid drying of the sample. The spot should be as small and uniform as possible. Handle the paper by its edges / corners.

Fold the filter paper into cylindrical shape and stitch it. Saturate the chromatographic chamber with the vapours of solvent mixture. Keep the paper inside the chamber with its edge dipping in the solvent. The sample spots should be well above the solvent level. The solvent moves through the paper by capillary rise. The different amino acids depending upon their polarity and solubility get separated. After the solvent has moved to about 4 cm below the top edge of the paper remove it. Mark the distance moved by the solvent on paper and air dry it. Spray with ninhydrin and then keep in a oven at 100°C for a few minutes. The amino acid spots appear purple in colour and mark them with a pencil. Measure the distance travelled by the solvent from the sample spots. Measure the distance travelled by the solute (amino acids) and calculate the Rf value.

$$\text{Rf value} = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

Compare the Rf values of amino acid spots of unknown sample with that of standard amino acids. The amino acids may be quantitatively estimated by measuring the intensity of the purple coloured spots in a colorimeter at 540 nm.

Result

1. Rf value of the given amino acid is ……..

2. The given sample was found to contain ……..

Things to learn

1. *What are other solvents used for separation of amino acids?*

2. *Will the Rf value be same for different solvent systems?*
3. *Why proline give yellow spot with ninhydrin?*

Exercise.12

Separation Of Sugars Through Thin Layer Chromatography

Ex. No. 12

Date : ……..

Thin Layer Chromatography (TLC) is an easy technique to adopt for separation, identification and characterization of unknown organic compounds. A variety of small molecules like amino acids, sugars, organic acids, lipids, etc. are separated by this technique. The greater advantage of TLC is the speed at which separation is achieved. When volatile solvents are used the time required to effect separation is only about 30 min. and with non-volatile solvents it is seldom longer than 90 min.

Principle

The general principle involved in TLC is similar to that of column chromatography, i.e., adsorption chromatography. In the adsorption process, the solute competes with the solvent for the surface sites of the adsorbent. Of course, the partition effect in the separation is also not ruled out. The adsorbent normally used, contains a binding agent such as calcium sulphate, which facilitates the holding of the adsorbent to the glass plate.

Reagents

- Glass plate (20 x 20 or 20 x 10 cm)
- Glass tank with lid
- Spreader
- Developing solvents. Differ with group of compounds to be separated.
- Adsorbent: Silica gel G / alumina
- Sample should be extracted following the procedures indicated for each group of compounds. For e.g., extraction with 80 per cent alcohol for amino acids and sugars.

- Standards
- Spraying agent. This also differs as for the group of compounds of interest.

Procedure

Place dry, clean glass plates (5 Nos. 20 x 20 cm) on the plastic base-plate over a plane surface.

Prepare a slurry of the adsorbent in water (sometimes buffer) in the ratio 1 : 2 (W/V). Stir the slurry thoroughly for 1 – 2 min. and pour into the applicator positioned on the head glass plate. Coat the slurry over the glass plates at a thickness of a 0.25 mm for qualitative analysis by moving the applicator at a uniform speed from one end to the other. Leave the plates to dry at room temperature for 15 – 30 min.

Heat the plates in an oven at 100 – 120°C for 1 – 2 h to remove the moisture and to activate the adsorbent on the plate. The dried plates in a rack can be stored in a desiccator over silica gel to prevent moisture absorption.

Sample application

Leave 2.5 cm from one end of the glass plate and at least one inch equal distance from the edges. Apply the sample and standard by means of a micropipette or syringe as small spots. All spots should be placed at equal distance from one end of the plate. See that the adsorbent does not flake off at the sample application point. Measured volumes are applied for quantitative estimation. Sample application can be done repeatedly for a more concentrated sample spot.

Developing the chromatogram

Pour the developing solvent into the tank to a depth of 1.5 cm. Allow it to stand for at least an hour with a cover plate over the top of the tank to ensure that the atmosphere within the tank becomes saturated with solvent vapour. This is called equilibration. After equilibration, remove the cover plate, and place the thin layer plate (sample applied) vertically in the tank so that it stands in the solvent, with the spotted end dipping in the solvent. Replace the cover plate. The separation of the compounds occurs as the solvent moves upward. Develop the chromatogram at constant temperature in order to avoid anomalous solvent running effects.

Once the solvent reaches the top of the plate, remove it from the tank. Dry and proceed for the identification of the separated compounds.

Two dimesional

In order to improve the resolution of a particular separation, the two dimensional chromatography may be used. Here, separation of the compounds is done in two different solvent systems. Apply the sample as a single spot at one corner of the plate. Develop in the first solvent system, remove from the tank and dry. Again develop in the second solvent system in the direction at right angles to the first development.

Calculation

If the commercially available adsorbent contains a fluorescent dye, the separated compounds will show up as blue, green or black spots when viewed under UV light. These areas can be scrapped, eluted with a suitable solvent, and quantitative estimations of the separated compounds can be carried out. When such a dye is not used in the adsorbent, one of the following methods of identification can be followed.

i. Spray with 50 per cent sulphuric acid and heat. This will result in most compounds getting charred and show up as brown spots.
ii. Examine the plates under UV light which show the locations of UV absorbing or fluorescent compounds.
iii. Expose to iodine vapour for unsaturated compounds.
iv. Spray with ninhydrin for amino acids.
v. If the compounds are radioactive, the plates may be subjected to autoradiography and can be detected as dark spots on X-ray film. Alternatively, a radiochromatogram scanner may scan the plate.

Preparative tlc

The TLC technique can also be used for the isolation of separated compounds and in that case, it is called as preparative TLC. Instead of a thin layer, with a thick layer (up to 5 mm) of adsorbent coated, a greater quantity of the sample can be applied onto the plate. After separation, the area of the separated compound is scrapped off, eluted with a suitable solvent and recovered in a relatively pure form.

In preparative TLC, usually the sample is applied as a streak rather than a spot, for, a large quantity can be applied. The compounds are also separated as series of bands which may be scrapped off and eluted.

Result

1. The Rf value of the given compound is ……..
2. The given sample was found to contain ……..

Things to learn

1. *Name the solvent system for separation of organic acids.*
2. *Why sulphuric acid is used as spraying agent for identification of carbohydrates.*

Exercise.13

Laboratory requirements, Equipments and Apparatus in Plant Tissue Culture

Ex. No.13

Date: ……..

Plant tissue culture is the culture of plant seeds, organs, tissues, explants, cells or protoplasts in a nutrient medium under sterile conditions. A tissue culture laboratory must contain the following common facilities, regardless of the specific purpose.

1. A general washing and drying area
2. A media preparation, sterilization and storage area
3. An aseptic transfer area
4. Environmentally controlled incubators or culture rooms
5. An observation/data collection area

Washing Area

The washing area should contain lead lined large sinks (to resist acids and alkalis), draining boards and racks and have access to demineralized/distilled/double distilled water. Space for hot air ovens, racks, automated dishwashers, acid baths, pipette washers & driers and storage cabinets should also be available in the washing area.

Media Preparation Area

The media preparation area should have ample storage space for the chemicals, culture vessels and glassware required for media preparation and dispensing. Bench space for hot plate stirrers, pH meters, electronic balances, water baths and media-dispensing equipment should be available. This room should also have access to distilled and double distilled water, Bunsen burners with a gas source, refrigerators and freezers for storing stock solutions and chemicals, a microwave or a convection oven and an autoclave or domestic pressure cooker for sterilizing media,

glassware and instruments. In preparing culture media, analytical grade chemicals should be used and good weighing habits practiced. Insuring accuracy and preparing a complete checklist even for a simplest media is must. The water used in preparing media must be of the utmost purity and highest quality. The tap water is unsuitable because it may contain cations (ammonium, calcium, iron, magnesium, sodium etc.,), anions (bicarbonates, chlorides, fluorides, phosphates etc.,), microorganisms (algae, fungi, and bacteria), gases (oxygen, carbon dioxide, nitrogen) and particulate matter (silt, oils, organic matter). Water used for plant tissue culture should meet, at a minimum, the standards for type II reagent grade water i.e., is free of pyrogens, gases and organic matter and have an electrical conductivity less than 1.0 μmho/cm. This can be achieved by treating the water with deionization treatment or glass distillation procedure. Since all new glass may release substances that affect the composition of the medium, it is recommended that all new glassware to be filled with water, autoclaved twice with detergent, washed and rinsed between washes before being used for tissue culture.

Transfer Area

It is advisable that a laminar flow hood or sterile transfer room be utilized for making transfers. Within the transfer area there should be a source of electricity, gas, compressed air and vacuum. The most desirable arrangement is a small dust free room equipped with an overhead ultraviolet light and a positive pressure ventilation unit. The ventilation should be equipped with a high-efficiency particulate air (HEPA) filter. A 0.3 μm HEPA filter with 99.97-99.99% efficiency works well. All surfaces in the room should be designed in such a manner that dust and microbes do not accumulate and the surfaces can be thoroughly cleaned and disinfected. In laminar air flow hood chamber, the air should circulated through HEPA filter and directed downward (vertical flow unit) or outward (horizontal flow unit) over the working surface which prevents settling of particulate matter on the working surface. The simplest type of transfer area suitable for tissue culture is an enclosed plastic box commonly called a glove box. This type of culture hood is sterilized by an ultraviolet light and wiped down periodically with 95% ethyl alcohol when in use.

Culture room

All types of tissue culture should be incubated under conditions of well-controlled temperature, humidity, air circulation and light quality and duration. These environmental factors may influence the growth and differentiation process directly during culture or indirectly by affecting their response in subsequent generations. Protoplast cultures, low density cell suspension cultures and anther cultures are particularly sensitive to environmental cultural conditions. The culture room should have temperature between 15° and 30°C, with a temperature fluctuation of less than ± 0.5° C; however, a wide range in temperature may be required for specific experiments. The room should have an alarm system (to indicate when the temperature has reached preset high or low temperature limits) and continuous temperature recorder (to monitor temperature fluctuations). The temperature should be constant throughout the entire culture room (i.e. no hot or cold spots). The culture room should have enough fluorescent lighting to reach the 10000 lux; the lighting should be adjustable in terms of quantity and photoperiod duration. Both light and temperature should be programmable for a 24 hr period. The culture room should have fairly uniform forced-air ventilation and a humidity range of 20-98% controllable to ± 3%.

Equipments, Apparatus and Accessories

The following items are commonly found in laboratory for *in vitro* propagation of plant materials.

Equipment description	Function
Water purification system: Water should have a resistivity of at least 200000 ohms/cm and a conductivity of 5.0 mmhos/cm	Purification of water for media preparation.
Electronic Balance: (0.01g readability; 200 g minimum capacity)	Measuring out biochemicals and media.
pH meter:	Measurement and adjustment

(range 0 – 14; ±0.01 accuracy; automatic temperature compensation 0 - 60°C; one or two point calibration)	of pH
Hot plate stirrer: (7" X 7" ceramic top; variable heating range from ambient to 400 °C; variable stirring speed from 50 – 150 rpm; chemically resistant)	Mixing and heating media and stock
Magnetic stirring bars: Teflon coated, leak proof.	Used for mixing solutions and in media preparation
Thermometer: -20 to 150 °C temperature range	Measuring temperature of liquids and culture room
Timer: Electronic, countdown timer alarm, stopwatch feature	Timing the sterilization process and for general lab use.
Refrigerator/freezer: Capable of maintaining a refrigerator temperature of 0 – 5 °C with a freezer temperature of approximately 0 to -20°C	Storage of stock solutions, media and hormones
Laminar flow transfer hood: Incoming air should be HEPA filtered to remove 99.99% of particles larger that 0.3 μm	Provide sterile atmosphere to transfer cultures
Sterilizer: Pressure cooker – operates between 116-126 °C; 10-20 psi. Autoclave – operates at 120 °C, 20 psi and has lid with pressure gauge, safety valve (to release the pressure)	Sterilization of media and instruments
Nylon membrane filters	Sterilization of heat liable compounds
Isopropyl alcohol or ethanol	Used to sterilize instruments

	and work areas
Aluminum foil	Used to wrap instruments prior to sterilization, cover vessels
Beakers (250, 1000, 2000 and 4000 ml): All the glassware should be made up by borosilicate	Mixing solutions and media
Bottles (100, 250, 500 and 1000 ml)	Storage of media stocks etc.,
Culture tubes	Used in Starting cultures
Erlenmeyer flask (1000, 2000, 3000 and 4000 ml)	Mixing and storage of media
Forceps: Dissecting; 10" length, serrated, stainless steel	Transferring tissue
Graduated cylinders (glass or polypropylene) 10, 25, 50, 100, 500 and 1000 ml	Preparation of stock solutions
Pipettes (1, 5, 10, 25 ml)	Measuring out stock solutions
Parafilm	Wrapping culture vessels
Gloves (should provide protection up to 350 °C)	Safely removing hot items from autoclave
Scalpel blades with handle	Cutting explants
Scoop: Should be big plastic scoop	Measuring large volume of biochemicals
Spatula: stainless steel	Measuring small to medium amounts of biochemicals
Weighing boats: Plastic with different sizes	Measuring chemicals

Exercise.14

Preparation of stock solutions for MS medium, MS Medium Preparation and Sterilization Techniques

Ex. No.14

Date: ……..

The degree of success in any tissue culture work mainly depends on the choice of nutritional and hormonal factors. A defined nutrient medium consists of inorganic salts (Macro, Minor and Micronutrients), a carbon source, vitamins and growth regulators and water. A solidifying agent is added in case of preparation of a semisolid medium. All these compounds fulfill one or more functions in the *in vitro* growth of plants. The minerals present in plant tissue culture media can be used by the plant cell as building blocks for the synthesis of organic molecules or as cofactors in enzymatic reactions. The ions of the dissolved salts play an important role as counterion in the transport of ionized molecules by the plant, in the osmotic regulation and in maintaining the electrochemical potential of the plant.

Nitrogen, Sulfur and Phosphorous are components of proteins and nucleic acids. Magnesium and many micro elements form essential parts of enzymes and cell organelles and are therefore important in catalyzation of various reactions. Calcium and Boric acid are mainly found in the cell wall and especially calcium has an important role in the stabilization of biomembranes. Potassium and Chloride, on the contrary, are important in the osmotic regulation, for maintainance of the electrochemical potential and for the activation of a large number of enzymes. Sucrose and Glucose are essential carbon sources. Plants generally grown *in vitro* have a requirement for vitamins like Thiamine, Nicotinic acid and Pyridoxine. Some amino acids were found to be facilitating plant regeneration E.g. L-Glutamine. Auxins and Cytokinins are the two groups of growth regulators frequently used in tissue culture work. These plant hormones are synthesized in one tissue and act in the same tissue or transported to another tissue to activate certain regulatory processes.

Gibberellins, Abscisic acid and ethylene are other growth regulators used for specific purposes. Natural complexes such as coconut milk, fruit juices (orange juice, tomato juice), yeast extract, malt extract, potato extract and fish emulsion have also been found effective in tissue culture.

Murashige and Skoog (MS) medium is the most used tissue culture medium and many variants have been developed. The medium is derived from White's medium and originally developed for the cultivation of *Nicotiana tobaccum* calli. Compared to the Whit medium, the concentration of all the ingredients is increased. This increase in concentration stimulated the growth of the calli. However, due to the high concentration of minerals, MS medium is a very rich and saline medium and can be too salty to certain plant species.

Preparation of Stock Solutions

Stocks are convenient for frequent preparation of working solutions. It saves time since it doesn't require repeated weighing of same ingredients whenever the medium is prepared. All the stock solution should be properly labeled and the label should contain,

1- Name of Stock Solution

2- Concentration of stock (E. g. 10 X or 50 X)

3- Date of preparation

4- Initials of the person who prepared it.

Points To Be Remembered

1. The final concentration of the solution used in the media should be known before preparing a stock. The stocks should be convenient multiples of the final concentration (e. g. Macronutrient's stock solution is prepared as 10 X concentration). This is also decided by the quantity of the chemical required (e. g. Vitamins are prepared in 100 X concentrations).
2. Stock solutions should be prepared in double distilled water.

3. Stocks solutions of hormones and vitamins should be prepared in 95% Ethanol. This prevents contamination and eliminates the need for sterilization since alcohol is a sterilant. The use of more than 5 ml Ethanol/litre of medium is not advised as it may have detrimental effects on the growth of tissues. Vitamin stocks can also be prepared by dissolving the desired vitamin into 50 ml double-distilled water (DDH2O). Transfer the vitamin solution to a 100 ml volumetric flask and bring the volume to the mark with DDH2O. Vitamins are weighed one by one and dissolved in demineralized water.
4. Do not prepare stocks of myo-Inositol or sucrose (sugar).
5. Stock solutions are generally stored at 4°C and should be checked by visual inspection before each use. This prevents the use of contaminated solutions. Additionally, stocks of vitamins and growth regulators should not be stored for prolonged periods of time as they may not work as effectively. It is suggested that generally prepare stocks once every two months.
6. For organic salts, the chemicals are dissolved in distilled or high purity de mineralized water. Only one compound is added at a time to avoid precipitation. Dissolving the calcium salts separately will prevent precipitation of salts.
7. The Iron stock (Na-Fe-EDTA solutions) should be protected from light by storing in amber colored bottles. The sodium ferric salt of EDTA also can be obtained from commercial source.
8. KI is weighed and dissolved separately. This is highly light sensitive and hence stored in amber coloured bottles in the refrigerator.
9. The amino acids should be dissolved separately and filter sterilized before being added to the autoclaved medium.
10. Antibiotics is soluble in water and should be made fresh and be added to the medium after autoclaving by filter sterilization.

Table 1. Preparation of Stock Solutions for MS medium

Ingredients	Final composition in the medium (mg/l)	Stock solution (W/V)	Volume of the stock to be taken per litre of medium
Macro Nutrients (10X)	1650		**100 ml**
NH_4NO_3	1900	16.50 g	
KNO_3	440	19.00 g	
$CaCl_2\,2H_2O$	370	4.400 g	
$MgSO_4.\,7H_2O$	170	3.700 g	
KH_2PO_4		1.700 g **in 500 ml**	
Minor Nutrients (100X)	22.3	2.23 g	**2.5 ml**
$MnSO_4\,4H_2O$	8.6	0.86 g	
$ZnSO_4\,4H_2O$	6.2	0.62 g **in 250 ml**	
H_3BO_3			
Micro Nutrients(100X)	0.25	22.3	
$Na_2MoO_4.\,2H_2O$	0.025	8.6	
$CuSO_4\,5\,H_2O$	0.025	6.2	
$CoCl_2\,6\,H_2O$			**1.0 ml**
Iron Stock (50X)	37.25	22.3	**2.5 ml**

Na_2EDTA $FeSO_4\ 7H_2O$	27.85	8.6 6.2	
KI (100X) KI	0.83	0.083 g **in 250 ml**	**2.5 ml**
MS Vitamins (100X) Nicotinic Acid Pyridoxine.HCl Thiamine. HCl Glycine	0.5 0.5 0.1 2.0	50 mg 50 mg 10 mg 20 mg **in 100 ml**	**1.0 ml**

Solvents for Plant Growth Regulators

- Generally, Cytokinins are readily soluble in acidic solutions (1 or 0.5 N HCl), while auxins are soluble in basic solutions (1 N NaOH).
- Dissolve the growth regulator in 2.5 ml of solvent, heat gently and bring to volume with water or ethanol. Once dissolved, stocks of growth regulators are brought up to volume using water (tissue culture grade), but can also be used with 70% or 95% ethanol (ETOH) for sterilizing purposes.
- Adjust the pH of the stock solution to pH 5.0 before storage at 4°C. The 2, 4-D is usually dissolved in dimethyl sulfoxide (DMSO), even though it is also soluble in ETOH. Caution should be taken when using DMSO since it may have adverse effects at high concentrations and can penetrate the skin readily.
- Gibberellic acid should be dissolved in water and adjust the pH to 5.7. GA_3 is thermo unstable and must be filter sterilized.
- Abscisins: ABA is heat and light sensitive.
- **Others** Thidiazuron (TDZ) is dissolved in DMSO and kept as a stock in diluted DMSO solution. Silver nitrate ($AgNO_3$) should be dissolved in 5 ml of

95% ethanol, stirred, heated gently and the volume is made up with distilled water. Silver nitrate must be filter sterilized.

- **Coconut water (5-20%)** This is prepared from green-ripe coconuts. A hole is drilled through a germination pore and the water is drained through coarse filter. The water from several nuts is combined and kept frozen.

Note

Different kinds of media compositions are now available in the form of powders, but expensive. It is essential that the materials in the powder are completely dissolved thus will be available to the cells. The kinds and quantities of stock solutions vary with the operation and preferences and stored in refrigerator. The required quantity is pipetted out based on the concentration of each of the stock and media volume.

The general formula is,

$$\text{Volume of stock required} = \frac{\text{Required Concentration} \times \text{Media Volume}}{\text{Concentration of Stock Solution}}$$

Sterilization Techniques

Successful tissue culture procedure relies on the maintenance of aseptic or sterile conditions. Since the culture medium contains sugar (as a carbon source), it attracts a variety of microorganisms. The microbes grow faster than that of the cultured tissue in medium and they ultimately kill the plant cells. Hence, complete aseptic conditions around the culture environment are necessary.

Sources of contamination

1. Medium

The microorganisms may be present in the nutrient medium at the time of its preparation which can be destroyed by proper autoclaving. The medium can be

sterilized in an autoclave at 15 lb pressure at 121°C for 20 minutes or in a pressure cooker. For large volumes, the time should be increased (30 minutes for 500 ml and 40 minutes for 1000 ml). Filter sterilization is recommended for heat labile compounds. Filter sterilizing of complex materials such as coconut milk will require at least two different filters. After an initial coarse filter, use a pre filter of 0.4 µm and a final filter of 0.22 µm size.

2. The Explant

The plant part may carry microorganisms and so the plant part should be surface sterilized by using surface sterilants. Plant materials can be sterilized in different ways.

1. The most convenient procedure is to produce the explant material under sterile condition i.e. from aseptically germinated seedlings. The explant to be used are then removed and used immediately. This method prevents damage, especially, when exposed to the chemical sterilants.

2. Liquid detergents can be used in initial washings. A wetting agent like Tween-20 can also be included to increase the efficiency of sterilization procedures.

3. Explants can be collected from the field and sterilized using chemical sterilants such as

i. Sodium hypochlorite (NaOCl) (0.025-0.25%). Diluted household bleach can be used which contains 5.25% NaOCl. It is less expensive and effective.

ii. Calcium hypochlorite (CaOCl) (20-100% dilution of saturated solutions). This cause less damage but tends to precipitate. So, sterilization solutions must be filtered and decanted prior to use.

iii. Hydrogen peroxide (H_2O_2) (3-10%). Effective and easier method than NaOCL or CaOCl.

iv. Bromine water (1-2%) Silver nitrate (1%) Mercuric chloride (0.11-1%%) and Chlorine gas can also be used for sterilization of seeds, tubers and woody species,

4. Ultrasonic bath is particularly useful for sterilizing buds and woody tissues which have many small surface crevices and cracks.

Tween 20 or Triton X-100 (0.05%) can be added to the chemical sterilization solutions to ensure contact of the tissue with the sterilants. Stirring the tissues during this process will facilitate good surface contact. Conducting sterilization processes in vacuum helps in removing air bubbles and provides efficient sterilizations. After surface sterilization, a minimum of three sequential rinses with sterile distilled water are recommended to remove any remaining chemical sterilizing agents. It is always better to germinate single/few seeds per container as a single contaminant seed may contaminate all the remaining seed in culture.

Hard coated seeds of wild species require scarification before sterilization and germination. Procedures vary greatly depending upon the nature of the explants. Tender and sensitive explants must be treated more gently. Concentration and/or time can be increased if contamination is a problem. Standardization is required for each and every explant, genotype and crop species.

Trouble shootings

If tissue turns brown and dead, tissues are over sterilized. Reduce length of sterilization/concentration of sterilizing agent. If tissues are highly contaminated, sterilization needs to be more stringent. Mercuric chloride can be more effective than the hypochlorite treatments. In case of browning of explants due to exudation of phenolic compounds, treat the explants with antioxidants such as L-Ascorbic acid-free acid 0.1 g/1, citric acid-free acid anhydrous 0.15g/1, L-Cysteine HCL 0.5g/1 which are commonly used for checking browning problem.

3. The Working Area

Surfaces can be decontaminated using a solution of 70% Ethanol (ETOH). Additionally, the use of bactericidal lamps (UV light) can help disinfect the work area. Wipe down the surface of laminar airflow hood with alcohol /iso-propanol /other disinfectant before starting the procedure. Utensils can be also sprayed with ETOH solutions prior to use, but should be flame sterilized when used (metal or glass utensil only).

Detection of contamination

Contamination in the media is mainly due to spore transfer or contact with incompletely sterilized media, solutions and surfaces. Bacterial contaminations are recognized by turbidity in liquid media, a growth with coloration and sometimes by unusual odors. Yeast growth often appears as heavy 'milky' turbidity in liquid media and has a distinctive odor. Fungi form mycelia or balls in liquid media. Mites and thrips can also found in the media storage room which may carry several microbial contaminants.

Techniques for sterile Transfer hoods

1. Wash the hands with soap and water before starting any work.
2. Set up all containers, media, and utensils in transfer hood in such a way that it will not disturb airflow patterns (if so, it may introduce non-sterile air).
3. Dip the utensils in 95% ETOH and pass it through the flame. Don't let to get ETOH near your hands. Allow a few seconds for the utensil to cool and test if it is cool enough for use by touching a section with agar. Hot utensils may damage tissue and should be avoided. Also, be careful not to place a hot utensil in the ETOH solution as it may catch on fire.
4. Bottle and flask mouths should also be flame sterilized before and after pouring media. Flame the mouth of the vessel for a few seconds by passing it over a flame several times. This will prevent contaminants introduced into the medium from the edges.
5. When working under sterile conditions, avoid using jewels (e.g. rings, bangles). Contaminants are carried by particles, such as dead skin or fallen hair and therefore avoid leaning over or reaching over an open culture flask, petridish or other container with cultures when working in the laminar airflow hood. Move in a lateral direction within the hood or from the front toward the back of the hood.
6. Talking in hood should be avoided.
7. Cover the containers as quickly as possible after an operational step is completed and wipe surface clean with disinfectant after finishing the

experiment. A stainless steel working surface is the most durable and easiest material to keep clean.

8. Label each vessel with date and essential coded information that identifies the culture: date of inoculation, name of the crop, name of the researcher, previous sub-culturing details, stage of culture (such as initiation, multiplication, elongation, rooting etc.

Techniques for sterilization of seeds

Seed + Liquid detergent – 3 Min. (in a shaker)

↓

Rinse in tap water to remove soap residue

↓

70% ethanol –2 Min

↓

20-40% commercial bleach, 2-3 drops Tween 20

(or) 1% Sodium hypochlorite (NaOCl) – 15-20 Min.

↓

Rinse 5 times in sterile water

↓

Germinate in 1/10 strength basal medium (MS/B5) with 1/6 strength of sucrose (0.5%) (or) on filter paper in Petri dishes

Techniques for sterilization of bud, leaf and herbaceous stem sections

Submerge tissue in 70% ethanol – 1-3 Min.

↓

20% Commercial bleach / 1% sodium hypochlorite (NaOCl) / 0.1% $HgC1_2$ – 5 Min.

↓

Rinse 4-5 times in sterile water

Techniques for sterilizing tubers, roots and bulbs

Wash with detergent in running tap water (to remove the adhering soil particles)

↓

70% ethanol—1-3 Min.

↓

20% Commercial bleach or in 1% sodium hypochlorite (NaOCl) – 20 Min. (repeat if necessary)

↓

Rinse 4-5 times in sterile water

Exercise.15

Inoculation of Explant – Shoot Tip, Meristem Tip and Embryo

Ex. No.15

Date: ……..

Shoot tip (rhizome bud) culture in banana

Conventionally bananas (*Musa* spp.) are propagated using suckers that arise at the base of the main pseudo stem. However, the planting material is always in short supply and frequently contaminated with pathogens. *In vitro* techniques offer an effective and alternative strategy for mass multiplication of virus free bananas on commercial scale. This strategy is much useful in case of slow multipliers and shy suckering types. Shoot tip culture is widely used for rapid clonal propagation in which larger (5-10 mm) explants are used in other plant species. If shoot tips of up to 1 μm is used then it is meristem culture. Therefore, in most cases meristem tip culture are essentially shoot-tip cultures.

Media for Rhizome bud culture

MS medium + Benzyl amino purine (BAP) - 5 mg/1

(For initiation and multiple shoot induction)

Materials Required

1. Banana suckers
2. Sterile knife
3. Antioxidant solution:
   Ascorbic acid 100 mg

   Citric acid 150 mg in one litre of sterile water
4. 70 % ethanol
5. 0.12 % mercuric chloride

6. Sterile double distilled water

Protocol

Collect small suckers from field grown bananas and wash it in running tap water to remove adhering soil residues.

↓

The older leaves and the outer leaves are trimmed off carefully by unwhorling of leaf sheath and a small portion of rhizome.

↓

The trimming is continued till the shoot tip measures 4 cm long with a rhizome length of 3 cm and a width of 2.5 cm.

↓

To prevent the oxidation of phenolic compounds, the trimmed buds are stored in antioxidant solution till the buds are taken to laminar flow chamber for inoculation.

↓

Initial sterilization is done with 70 % ethanol for 30 seconds followed by 0.12 % mercuric chloride for 10 minutes.

↓

The buds are again washed several times with autoclaved distilled water to remove traces of sterilants.

↓

Give a final trimming to remove the outermost whorl of the shoot tip and rhizome portion.

↓

A vertical cut is given at the tip (to arrest the apical dominance) and the buds are inoculated in the semi-solid medium prepared for multiple shoot induction.

Meristem tip culture

Cultivation of axillary or apical shoot meristems is known as meristem culture. Meristem culture involves the development of an already existing shoot meristem and subsequently, the regeneration of adventitious roots from the developed shoots. It usually does not involve the regeneration of a new shoot meristem. Shoot apical meristem lies in the shoot tip beyond the youngest leaf or first leaf primordium. Thus a shoot tip (usually measures 100-500 μm) contains 1-3 leaf primordia in addition to the apical meristem. Meristem culture is widely practiced for production of virus free plants.

Materials required

1. Sodium hypochlorite + Tween 20
2. Absolute ethanol
3. Solid MS medium
4. Dissection microscope

Protocol

Collect the young stems along with the growing buds and excise stem segments containing at least one node from the donor plant.

↓

Remove mature and expanding foliage to expose the terminal and axillary buds.

↓

Cut segments to 4-cm lengths, and pre sterilize by immersion in 100% ethanol for 30 sec.

↓

Sterilize by immersing the segments in the sodium hypochlorite solution, with added detergent, for 8 min.

↓

Rinse the tissues three times in sterile distilled water.

↓

Mount the stem segment on the stage of the dissection microscope, and use the tips of hypodermic needles to dissect out progressively smaller, developing leaves to expose the apical meristems of the bud. Leave few youngest leaf primordia.

↓

Excise the explant tissue that should comprise the apical dome and the required number of the youngest leaf primordia

↓

Transfer the explant directly to the growth medium and close the culture vessel

↓

Keep it in incubation room

↓

If the explant is viable, the enlargement, development of chlorophyll, and some elongation will be visible within 7-14 days. Maintain the developing plantlet *in vitro* until the internodes are sufficiently elongated to allow dissection into nodal explants.

Embryo culture

Embryo culture is removal of young embryos from developing seeds and placing them in suitable nutrient medium to obtain seedlings. The cultured embryos do not complete development, but germinate prematurely to give rise to seedlings. Young embryos need to be isolated with their intact suspensors as it provides gibberellins to the developing embryo. Advantages of embryo culture are:

i. Overcoming seed dormancy and seed sterility
ii. Rescuing incompatible hybrid crosses
iii. *In vitro* germination of under-developed embryos.

Hybrid Embryo Rescue

Distant crosses may fail due to several reasons (inability of pollen to germinate, failure of pollen tubes to grow or degeneration of endosperm). When embryo fails to develop, embryo culture is used to recover hybrid plants. This is called as hybrid embryo rescue. Hybrid embryos are difficult to isolate and in most cases immature embryos are used. The same procedure can be applied to hybrid embryo rescue when such crosses are available in any species.

Materials required

1. Immature Soybean seeds.
2. MS medium

Protocol

Embryos at the heart stage or at the beginning of the cotyledon stage measuring 0.2-0.4. mm in length are ideal. The best morphological indicator for embryo developmental stage is pod width. For soybean pods are selected of size 3.0-3.7 mm in width, about 4-5 days after antithesis. Those pods contain seeds of 2.0-2.4 mm long embryos at the required stage.

↓

Seeds are generally surface sterilized just before dissection.

↓

The excision of an immature embryo embedded in liquid endosperm involves an incision at micropylar end of young ovule and the application of pressure at the opposite end to force the embryo out through the incision opening.

↓

After excision, larger embryos are transferred to culture medium with a pair of sterile forceps.

↓

Smaller embryos are handled with a dissection needle with its tip moistened or otherwise, Embryos will tend to stick onto surface moisture of the needle.

Exercise.16

Anther and Microspore Culture

Ex. No.16

Date: ……..

Anther culture is widely used for production of homozygous haploid plants. Androgenetic *in vitro* method is the culture of intact anthers. This is relatively simple and fast method and requires only minimal facilities. Alternatively, microspores can be mechanically isolated and cultured independent of the anther (called as pollen culture). These microspores represent a unique experimental material, since it is possible to isolate large numbers of single, almost synchronously developing cells, which will eventually undergo embryogenesis. Therefore, isolated microspores are considered as ideal target cells for *in vitro* selection and transformation.

Materials required

1. Rice panicles
2. 70 % ethanol
3. 0.1 % mercuric chloride
4. Sterile forceps, Scissors
5. Media
    a. MS+ 2,4-D 1.5 mg/1+NAA 0.5 mg/1 for callus induction
    b. MS+ BAP 2 mg/1 for shoot regeneration
    c. ½ MS+ NAA 0.5 mg-1+ IBA 1 mg/1 for *in vitro* rooting.

Protocol

Collect the flowers from the plants

↓

Surfaces sterilize with 70 % ethanol for one minute and in 0.1 % mercuric chloride for seven minutes.

↓

Rinse with sterile water for 4-5 times.

↓

Remove the individual spikelets and give a bottom 1/3 rd cut to remove the filaments.

↓

Using a bent forceps, hold the top 2/3 rd portion of the spikelet, and tap it on the mouth of the test tube. Avoid damage to the anthers.

↓

Arrange the anthers inside the test tube and incubate in dark for callus induction for 4-5 weeks at 25°C.

↓

After 4 weeks, assess the anther culture response by counting the number of responding anthers that gave rise to embryogenic callus.

↓

Transfer callus carefully to regeneration medium and incubate for about 3-4 weeks in the light (16 hr) at 25°C.

↓

Transfer plantlets to soil after *in vitro* rooting and determine the number of regenerates.

Microspore culture

Cut the spikelets and remove the anthers

↓

Blend in a micro blender with 20 ml of medium containing an osmoticum like 0.3 M mannitol

↓

Isolate microspores by blending twice for five seconds at low speed.

↓

Filter the crude microspore suspension through a 100 mM sieve and retransfer the material on the sieve back into the blender.

↓

Repeat the whole procedure three times with 10 ml of the above medium

↓

Transfer the whole extract into a 50 ml tube and centrifuge for 8 minutes at 3000 rpm (20°C)

↓

Remove the supernatant with a pipette. Do not pour off, since the micro pellet is soft and resuspend quickly.

↓

Resuspend the pellet in 8 ml of 19% maltose and transfer the suspension to a 10 ml tube.

↓

Carefully place the 1 ml layer of 0.3 M mannitol on top.

↓

Centrifuge for 10 minutes. The fraction of viable microspores is located in a band as the mannitol/ maltose inter phase.

↓

Collect the band carefully and transfer it to a 50 ml tube.

↓

Add 20-30 ml of 0.3M mannitol.

↓

Determine the total number of microspores with a haemocytometer. Additionally, viability of microspores can be determined with Fluorescein diacetate.

↓

Centrifuge the microspore suspension as before, remove the supernatant carefully and add liquid culture medium to final density of 2-5x 10^5 microspores/ml.

↓

Culture 1 ml aliquots in Petri dishes

↓

Keep cultures in dark at 26°C and add after 1-2 weeks, 1 ml of fresh liquid culture medium

↓

Determine the percentage of proliferation microspores after 2 weeks

↓

After 3-4 weeks, transfer the microspore derived aggregates onto a solid medium and culture at 26°C in the dark.

↓

Transfer the cultures to the light after one week for regeneration (16 hrs, 24°C)

↓

Sub culture at intervals of 2 weeks, and transfer shoots to regeneration medium

↓

Transfer regenerated plants to soil.

Exericse.17

Isolation of Bacterial Plasmid

Ex. No.17

Date: ……..

Plasmids are extrachromosomal, covalently closed circular, self replicating genetic material found in bacteria. They may exist independent or become integrated into the bacterial chromosome. There are several types of bacterial plasmids: F plasmids- responsible for conjugation, R plasmids- carry genes resistance to antibiotics, Col plasmids – code for colicins, the proteins that kill sensitive *E. coli* cells; they also carry genes that provide immunity to the particular colicin. Most of the *E. coli* plasmids are used as vectors. A vector is a DNA molecule that has the ability to replicate in an appropriate host cell and into which the DNA fragment to cloned (called DNA insert) is integrated for cloning.

Reagents required

1. LB (Luria Bertani) Broth:	pH 7.0
Yeast extract	5.0 g
Tryptone	10.0 g
Sodium chloride (NaCl)	10.0 g
Distilled water	1.0 litre
Ampicillin	50.0 µg/ml (Optional)

(Autoclave and store it at 4° C)

for LB agar, add 15 g of Agar per litre.

2. Suspension Buffer

Glucose	50.0 mM
Tris-HCl (pH 8.0)	25.0 mM
EDTA (pH 8.0)	10.0 mM

(Autoclave and store it at 4° C)

3. Lysis buffer

Sodium hydroxide (NaOH)	0.2 N
Sodium Dodecyl Sulphate (SDS)	1.0 %

(SDS should not be autoclaved and may be filtered through 0.45 µ filter and stored at room temperature)

4. Potassium acetate solution	3 M (pH 4.8)

Potassium acetate 5 M (pH 4.8)	30.00 ml
Glacial acetic acid	5.75 ml
Water	14.25 ml

5. TE buffer (pH 8.0)

Tris-HCl	10.0 mM
Ethylenediaminetetraacetic acid (EDTA)	1.0 mM

(Autoclave and store at 4 ° C)

6. Absolute ethanol (Keep it in -70° C freezer)

7. Ethanol 70.0 %

8. Phenol: Chloroform mixer 1:1

Protocol

Inoculate 2 ml of sterile LB broth with a single bacterial colony (*E. coli* DH5α having pUC or any other plasmid)

↓

Incubate the culture at 37 °C for 14-24 hrs in a shaker (150-200 rpm) or until the *E. coli* cells reach the OD_{600} ~ 4.0

↓

Take 1.5 ml of this culture into an Eppendorf tube and centrifuge at 10,000 rpm for 20 seconds

↓

Discard the supernatant and save the pellet

↓

Resuspend the pellet in 100 µl of ice cold suspension buffer and keep in ice for 5 minutes

↓

Add 200 µl of lysis buffer and mix by inverting several times and incubate the tube in ice for 5 minutes

↓

Add 150 µl of ice cold 3 M Potassium acetate solution, mix well and incubate on ice for 5 minutes

↓

Centrifuge at 12000 rpm for 5 minutes and transfer the supernatant to a fresh Eppendorf tube

↓

Add 0.5 ml of Phenol: Chloroform mixture and mix by inverting the tube several times

↓

Take out the supernatant and add twice the volume of ice cold absolute ethanol, mix and keep it at -20°C for 30-40 min.

↓

Centrifuge at 12000 rpm for 5 minutes. Discard the supernatant.

↓

To the pellet, add 1 ml of 70% ethanol.

↓

Centrifuge at 12000 rpm for 5 minutes. Discard the supernatant and air dry the plasmid DNA.

↓

Dissolve the plasmid DNA in 30 µl of TE buffer

↓

Store the plasmid DNA at -20°C until it is used.

Exercise.18

Plant DNA Isolation

Ex. No.18

Date: ……..

Isolation of plant genomic DNA is a routine procedure in any molecular biological experiments. The genomic DNA represents the blue print of the life in the plant species and it has information for all growth and development of the organism. Each species has its own protocol to isolate its genomic DNA. Isolation of good quality and required quantity of genomic DNA is absolutely essential in all the experiments concerned with DNA. Impurities such as protein, carbohydrates, RNA etc. will certainly affect the progress and reproducibility of the experiments. Care should be taken while isolating the DNA since DNAase present in the wet hands and in external environments degrades the DNA.

Materials

1. Leaf samples

   2g of fresh leaf samples

2. Cetyl Trimethyl Ammonium Bromide (CTAB) Extraction Buffer (100 ml):

CTAB	-	2% W/V
Tris HCl pH 8.0	-	100 mM
Sodium chloride	-	1.4 M
EDTA	-	20 mM

(Autoclave Tris, NaCl and EDTA and 2% CTAB should be added after autoclaving and this buffer is preheated before using this buffer).

3. Tris EDTA (TE) buffer:

Tris HCl (pH 8.0) - 10 mM

EDTA (pH 8.0) - 1 mM

Dissolve and make up to 100 ml with distilled water, autoclave and store at 4°C.

4. Ice cold Isopropanol

5. Chloroform: Isoamyl alcohol (24: 1 V/V)

6. Sodium acetate (3.0 M) pH 5.2 (Adjust pH with glacial acetic acid)

7. Ethanol (100% and 70%)

8. RNAase A -10 mg/ml; Dissolve RNAase A in TE and boil it for 15 minutes at 100°C to destroy DNAase and store at –20°C.

Protocol (CTAB method)

Transfer 2 g of leaf bits into prechilled mortar, frozen using liquid nitrogen and grind it to fine powder.

↓

The fine powder is allowed to thaw in the presence of 10 ml of pre-heated extraction buffer and incubate for 30-45 minutes at 65°C with occasional mixing.

↓

Add equal volume of Chloroform: Isoamylalcohol mixture (24:1 V/V) and mix by inversion for 1 hour.

↓

Centrifuge at 10,000 rpm for 20 minutes at room temperature.

↓

Transfer the clear aqueous phase to a new sterile tube. Add equal volume of ice cold Isopropanol and mix gently by inversion and then keep it in the freezer until DNA is precipitated out.

↓

Using blunt end tips, spool out the precipitated DNA into an Eppendorf tube.

↓

Air dry the spooled DNA after removing the supernatant by brief spin.

↓

Add 500 µl of TE to dissolve the DNA and then 10 µl of RNase and incubate at 37°C for 30 minutes.

↓

Add 500 µl of Chloroform: Isoamylalcohol mixture and centrifuge for 10 minutes.

↓

Transfer aqueous phase to another Eppendorf without disturbing the inner phase.

↓

Add 2.5 volume of absolute Alcohol and 1/10th volume of Sodium acetate and keep for overnight incubation.

↓

Centrifuge and Discard the supernatant.

↓

Use 500 µl of 70% and 100% ethanol subsequently to wash the DNA using centrifugation.

↓

Discard the Alcohol and remove the water residue from the DNA completely by air drying.

↓

Dissolve the DNA pellet in 150-250 µl of TE (depends on the pellet size) and store at 4°C.

Rapid isolation of plant genomic DNA

Excise a healthy leaf blade (about 2 cm long) and store it in Eppendorf and place it on ice.

↓

Cut the leaf tissue into half cm long and place it in a well of spot test plate or glass plate

↓

Add 400 µl of extraction buffer (Tris-HCL 50 mM, pH 8.0; EDTA 25 mM; NaCl 300 mM; SDS 1%).

↓

Grind the tissue using a thick glass rod as a pestle. Again add 400 µl of the extraction buffer, mix it and transfer into the Eppendorf.

↓

Add 400 µl chloroform: isoamyl alcohol, mix well, and spin for 30 seconds in microcentrifuge.

↓

Transfer the supernatant into another Eppendorf. Care should be taken in not disturbing the interface.

↓

To the supernatant, add 800 µl of absolute alcohol and mix gently.

↓

Spin the tube for 3 minutes in microcentrifuge with full speed and Discard the supernatant.

↓

Wash the pellet with 70% ethanol and air dry the pellet.

↓

Dissolve the DNA in 50 µl of TE (Tris-HCI 10 mM, pH 8.0; EDTA 1 mM) and then store at -20°C.

Exercise.19

Agarose gel electrophoresis

Ex. No.19

Date: ……..

Agarose is a polysaccharide consisting of 1, 3 linked β-D-galactopyranose and 1, 4 linked 3, 6-anhydro-α-L-galactopyranose. This basic agarobiose repeat unit forms long chains with an average molecular mass of 120, 000 Daltons and representing about 400 agarobiose units. During electrophoresis, water is electrolyted, which generates protons at the anode and hydroxyl ions at the cathode. The cathodal end of the electrophoresis chamber then becomes basic and the anodal end is acidic. The use of buffering system is therefore required when charged molecules are electrophoresed through a separation medium. The two buffers commonly used for DNA electrophoresis are Tris-Acetate with EDTA and Tris-Borate with EDTA. Because the pH of these buffers is basic, the phosphate backbone of DNA will have a net negative charge and consequently will migrate anodally.

Materials

a. Loading dye:

Glycerol	50% (V/V)
Bromophenol blue	0.5% (W/V)
Xylene cyanol	0.5% (W/V)

b. 10X TBE (Tris Borate EDTA) buffer:

Tris base	107.8 g
Boric acid	55.03 g
EDTA ($Na_2.2H_2O$)	8.19 g

(Dissolve in 800 ml of milli Q water filtered through 0.22μm filter paper and make up to 1000 ml and store at 4°C).

c. Ethidium Bromide

Protocol

Seal the open ends of the gel casting plate with cello tape and place it on a perfectly, horizontally leveled platform.

↓

Add 2% Agarose to 1X TBE and boil it till the agarose completely dissolved and then cool it to 50-60°C. **Ethidium bromide** was used as a staining agent at the final concentration of 1μg/ml of agarose solution.

↓

Pour the Agarose gel into the gel-casting tray; Place the comb properly and allow it to solidify.

↓

After solidification of the agarose, remove the comb and cello tape.

↓

Mix the DNA samples (10 µl) with 2.5 µl of agarose gel loading dye and load into the gel wells. 100 bp ladder (Bangalore Genei Pvt. Ltd., Bangalore**)** should also be loaded in one well as standard markers.

↓

Run the gel at 5 V/cm for 4-5 hours

↓

Visualize and document the bands in gel documentation system

Methylene Blue Staining

The major advantage of using Methylene blue as alternative to ethidium bromide is that it is much safer and cheaper to use, a non-mutagenic chemical, requires only ordinary light and the gel can be scored directly.

Protocol

After electrophoresis, immerse the gel in 0.025% Methylene blue for 20-30 min. (**Do not add ethidium bromide during the agarose gel casting**)

↓

Decant as much Methylene blue as possible using a funnel from the staining tray to a storage container (this can be reused).

↓

Rinse the gel in distilled water for several times. DNA bands become increasingly distinct as gel destains. Best results can be obtained after overnight destaining in a small volume of distilled water.

↓

The gel is viewed over light box and the bands are scored directly.

Exercise.20

PCR – RAPD/SSR

Ex. No.20

Date: ……..

RAPD markers are PCR-based markers generated by arbitrary primers. A single, short oligonucleotide primer, which binds to many different loci, is used to amplify random sequences from a complex DNA template. RAPD uses only a single primer which is not so in the case of PCR. It is usually 10mer rather than a 20mer and the aim is to amplify several segments of the target genome in a 'random' fashion. This depend upon the fact that 10mer sequences are sufficiently common in a large genome so that just by chance at several unpredictable locations, two primers will anneal sufficiently close to one another on opposite strands of the template to amplify the intervening region. The 10-base primer kits that have a (G+C) content of 60 to 70% cab be used and they have no self-complimentary ends.

The RAPD-PCR amplification process undergoes 35 repeated cycles.

a. DNA denaturation at 94°C

b. Annealing of primer to single stranded DNA at 37°C

c. Primer extension catalyzed by '*Taq* DNA polymerase' at 72°C.

The genomic DNA was extracted from the samples and purified. The purified DNA was tested for its intactness by electrophoresis. The DNA was quantified and diluted to a concentration of 25 ng/µl. Then the DNA was used for RAPD analysis. The cocktail for the amplification was prepared as follows using 0.2 mL PCR tubes.

Reaction mixture (18 µl) contains

Particulars	**Aliquot**	**Final concentration**
DNA 25 ng/µl	3.00 µl	50 ng

dNTPs (2.5 mM) (Bangalore Genei Ltd., Bangalore)	1.20 µl	200.00 µM
Primer (Operon Technologies Inc., CA, USA)	1.00 µl	0.60 µM
10x assay buffer	1.80 µl	1 X
Taq polymerase(Bangalore Genei Ltd., Bangalore) (3 units/µl)	0.18 µl	0.036 units
Magnesium Chloride	0.18 µl	26.6 µM
Sterile distilled H_20	10.64 µl	To make up the volume to 18 µl
Total	**18.00 µl**	

The reaction mixture was given a momentary spin for thorough mixing of the cocktail components. Then the 0.2 mL PCR tubes were loaded on to a thermal cycler. (Different models of thermal cyclers manufactured by companies such as MJ Research Inc, USA, Biorad, Eppendor are available at this center).

The thermal cycler is programmed as follows:

Profile 1: 94°C for 2 min – Initial denaturation

Profile 2: 94°C for 1 min – Denaturing

Profile 3: 37°C for 1 min – Annealing

Profile 4: 72°C for 1 min – Extension

Profile 5: 72°C for 5 min – Final extension

Profile 6: 4°C for infinity to hold the sample

Profile 2, 3 and 4 were programmed to run for 35 cycles.

Standardization of PCR reactions

1. The reagents and sterile water are divided into aliquots to minimize the number of sampling errors.
2. To avoid cross contamination, via the electrophoresis equipment, the gel combs and casting trays are washed using 3% acetic acid.
3. If there is any doubt about a critical result, the experiment can be again repeated until to get an unambiguous result.
4. Various control strategies has to be followed to carry out PCR reaction successfully.

Important controls used in the standardization of the PCR reactions were

- PCR in the absence of exogenously added DNA is used as negative control to check the DNA-free status of reagents and solutions.
- PCR with positive control DNA is used to check the completeness of PCR mixture (to check the quantity of essential components including $MgCl_2$ in the cocktail mixture).
- PCR with sufficient quantity of positive control DNA is used to amplify weak but consistent amplicons and to identify sensitivity and efficiency of PCR.
- Negative and positive controls are used to check for spurious background bands and reaction specificity and to identify PCR parameters (includes annealing temperature and number of cycles) that were suitable for amplifying expected products.

Exercise.21

Practical examination

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