CHAPTER II

Gene expression profile of O.mossambicus exposed to agrochemicals

GENE EXPRESSION

The increasing use of synthetic agrochemicals is escalating worldwide pollution risks. Agrochemicals are toxic and are generally designed to kill unwanted organisms, but when applied on land, they get washed into the surface water and adversely influence, the life of aquatic organisms (Woo *et al.,* 2010). Uses of these pollutants have posed potential health hazards and have become a major cause of concern for aquatic environment because of their toxicity, persistency and tendency to accumulate in the organisms (Joseph and Raj, 2010). The impact of these agrochemicals on aquatic organisms is due to the movement of pesticides from various diffuse or point sources which are posing a great threat to aquatic fauna especially to fishes, which constitute one of the major sources of protein rich food for mankind (Sharma and Singh, 2007).

Over the past 15 years, global analysis of gene expression (mRNA expression) has emerged as a powerful strategy for biological discovery. Gene expression is the process by which information from a gene is used in the synthesis of a functional gene product. These products are often proteins, but in non-protein coding genes such as transfer RNA (tRNA) or small nuclear RNA (snRNA) genes, the product is a functional RNA. The genome-wide analysis of gene expression has recently become viable due to the development of Polymerase chain reaction (PCR) techniques and oligonucleotide microarrays in many organisms. Gene expression is shaped by both genetic and environmental components, and can therefore be considered as a "molecular phenotype" (Ranz and Machado 2006) because the transcription rate of a gene varies among genotypes in such a way that it is a heritable phenotype (Schadt *et al.,* 2003; Gibson and Weir 2005; Whitehead and Crawford 2006; Roelofs *et al.,* 2009). Furthermore, gene expression provides novel insight into biological processes as it has the ability to uncover phenotypes, which would not readily be visible via traditional approaches for e.g. studies done on sentinel fish provided the information pertaining to the biological significance of contaminant exposure (Vidal-Dorsch *et al*., 2011). Among several techniques used for screening the gene expression,

the one with effective measure and widely used is PCR. The semi quantitative/quantitative PCR technique is effective to measure the absolute transcript and provides valuable quantitative information on gene expression from different sources and samples (Peters *et al*. 2004; Huggett *et al*. 2005; Nolan *et al*. 2006). It is a flexible and sensitive quantitative method worldwide (Bustin *et al*. 2000). The quantification of gene expression has many advantages over other techniques such as shortest time, low cost of reagents and rapid results.

Pesticides have their own specific gene expression profiles since they bind with low affinity to more than one receptor resulting in a complex gene activation pattern (Larkin *et al*., 2003). It is likely that competition for ligands and trans-acting factors might be playing a significant role in the activation of these molecular events. For an instance if a compound can bind to both the ER and AR, are both pathways induced? Or, does one pathway predominate? What happens with mixtures? Do the specific compounds in mixtures interact with each other or compete? The genetic apparatus of an organism can interact with genotoxicants in a variety of ways and an understanding of the cellular mechanisms involved in these interactions provide the researcher the opportunity to predict and possibly prevent contaminant-induced genetic damage in exposed populations (Akpoilih 2012). A way to begin to unravel this complicated system and to understand the mechanisms that might be involved is to use global, open-ended gene expression profiling experiments to determine the pathways that are affected. Current awareness of the potential hazards of agrochemicals in the aquatic environment has stimulated much interest in the use of fish as indicators / environmental biomarkers for monitoring the toxicity of agrochemicals which can act either as carcinogens, teratogens, clastogens or mutagens (Tom *et al.,* 2002-03; Akcha *et al.,* 2003; Verlecar *et al.,* 2006; Obiakor *et al.,* 2012; Ullah and Zorriehzahra 2015). Fish serves as a useful genetic model for the evaluation of pesticide toxicity and utilizing assays for detecting the genotoxicity caused by agrochemicals can help in formulating long term strategies for fish conservation program (Kapour and Nagpure 2005; Akpoilih 2012).

Genetic toxicology is an area of science in which the interaction of DNA-damaging agents with the genetic material is studied in relation to subsequent effects on the health of the organism (Amanuma *et al.,* 2000; Anitha *et al.,* 2000). Ecogenotoxicology (Genetic Ecotoxicology/Eco-toxicogenomics) is an approach that applies the principles and techniques of genetic toxicology

to assess the potential effects of environmental pollution in the form of genotoxic agents on the health of the ecosystem (Osman 2014). Toxicogenomics is a relatively new field that uses genomic research tools and techniques, such as DNA microarrays, and quantitative reverse transcription–PCR to determine how exposure to chemicals and chemical aggregates affect molecular pathways and biological processes at individual and population level (Carvan III *et al.*, 2008). There is growing evidence that genomic tools and techniques are effective for identifying fish genes involved in responses to individual toxicants and to toxicant mixtures. There is rich documented literature witnessing research on molecular level of different fish species showing ill effects of pesticides on genes and DNA levels (Vargas *et al.,* 2001; Renn *et al*. 2004; Sánchez-Bayo and Goka 2005; Çavas and Könen, 2007; Gadhiai *et al*.,2008; Yadav and Trivedi 2009; Ondarza *et al*., 2014; Dar *et al.*, 2016). Table 2.1 depicts the list of gene expression studies on various teleost with tools, application studied worldwide. Teleost species play important roles in toxicogenomic research, as experimental models they play key roles in research conducted in many fundamental areas of vertebrate biology (e.g., development, physiology, evolution, Toxicology).

Hence, the above literature and data suggest a fine linkage of gene expression with the effect of pesticide. In line of this, the present chapter deals with the gene expression profiling of candidate genes of hypothalamus Pituitary Gonadal axis (HPG), hypothalamus pituitary interrenal axis (HPI) and hypothalamus pituitary thyroid axis (HPT).

Group/species	Tools	Applications	Literature
Rainbow trout	Genomics	Applied research	Thorgaard *et al*. 2002
Bony fish	Genomics	Reviews applications	Clark 2003
Catfish	Genomics	Review	Liu 2003
Salmonids	Microarrays	Methodology	Rise *et al*. 2004
Cichlids	Candidate genes	Behavioral ecology	Fitzpatrick *et al*. 2005
Marine fishes	Genomics	Ecology & physiology	Hofmann *et al*. 2005
Fish	Microarrays	Methods and applications	Miller & Macclean, 2008
Atlantic salmon	Genomics	Tool development	Von Schalburg *et al*. 2008
Marine fishes	Genomics	Review of applications	Nielsen *et al*. 2009
Catfish	EST	Cold acclimation	Ju *et al*. 2002
African cichlids	Microarrays	Comparative genomics	Renn *et al*. 2004
Stickleback	EST	Developmental traits	Kingsley *et al*. 2004
Salmonids	Linkage maps	Recombination rates	Danzmann *et al*. 2005
Teleost fish	Sequence	Evolution of genes	Volff 2005
Atlantic salmon	Microarrays	Expression change in farmed fish	Roberge *et al*. 2006
Rainbow trout	Microarrays	Toxicant exposure	Hook *et al*. 2006
Chum salmon	Microarrays	Genetic stock ID	Moriya *et al*. 2007
Salmonids	EST	Gene duplication	Koop *et al*. 2008
Goby	Microarrays	Phenotypic plasticity	Gracey 2008
Atlantic salmon	Microarrays	Farmed and wild fish	Roberge *et al*. 2008
African cichlids	EST	Adaptive radiation	Salzburger *et al*. 2008
Aquaculture	Microarrays	Culture profiles	Zhang *et al*. 2009
Tuna	Microarrays	Endothermy	Castilho *et al*. 2009

Table 2.1: Depicts the tools with its applications used in various species of teleost.

Materials and Method:

Experimental design

Oreochromis mossambicus, commonly known as tilapia, having an average weight (25±2 g) and size (12±3 cm) was brought from the pure brooders of Baroda and were exposed to the period of acclimation in the laboratory conditions in a well aerated dechlorinated tap water for 10-15 days period. Commercial fish food was supplied to the fishes during entire experimental phase. Test animals were categorized into treated and control groups (10 animals in each group). $1/10^{th}$ of the LC_{50} values of all the selected agrochemicals (IMI, PE, CZ and MN) were considered for the sublethal dose for *O.mossambicus* (as describe in detail in chapter I). The exposure period for the treated group was 14 days. As all the agrochemicals were miscible in water, hence it was used as solvent for dissolving agrochemicals and making appropriate dose of it. Control group was kept in dechlorinated water without any treatment. 30% water was changed after every 24 hours and physicochemical properties of water were measured twice in a week.

Total RNA isolation and PCR Amplification

On 15^{th} day fishes were removed and washed with freshwater. Control as well as treated groups was euthanized by decapitation and blood was allowed to drain and organs (Hypothalamus, Liver, Gills, Kidney, Thyroid, Ovary and Testis) were dissected out. The aim of the study was to check the gene expression pattern of candidate genes hence total RNA was isolated by Trizol-Invitrogen according to the method of Peterson and Freeman (2009) and concentration was measured spectroscopically by Perkin elmer. cDNA was reverse transcribed from 50ng total isolated RNA using Thermo Verso cDNA synthesis kit (AB-1453/B) and PCR for the candidate genes was performed with their specific primers and with standardized condition i.e. denaturation was performed at 95^0C for 1 min, annealing was carried out for different primer according to their respective T_m for 30 sec and extension was carried out at 72^0C for 7 mins with 18srRNA as the reference gene. A total of 35 cycles were carried out for all the genes (described in materials and method) and finally the amplicon obtained were checked on 2% agarose gel and images were taken using ABI gel documentation system. Relative quantification analysis of the PCR products was done using Image J software.

SDS PAGE and Western blotting:

15% SDS PAGE gel was made for 30ug isolated protein for kisspeptin 1 and kisspeptin 2 and was subjected to western blotting analysis as describe in materials and method.

Statistical analysis

Statistical differences among treatment groups were tested using analysis of variance (ANOVA) and comparison of different exposure treated and control groups were performed using Prism 6.0 software. Linear regression analysis of individual genes with kiss 2 was performed to check if there is any significant association of one on the other in HPG axis using SPSS software version 21. R^2 was estimated for each combination as the ratio of regression sum of squares to total sum of squares. This was carried out because it is the one of the most useful method as it explains the dependency of the variables individually and determines their significance with greater accuracy.

Pathway representation:

Bioinformatic tools were used for representation of pathway and to investigate which pathways were being affected by the tested agrochemicals as describe in materials and method.

Results:

The one way ANOVA tested data revealed that significant alteration (*p<0.05) was found in various candidate genes of respective axis, which are represented as follows:

HPG axis:

PE exposure resulted significant (*p<0.05) down regulation of GnRH I expression, whereas an insignificant up regulation of *GnRH-I* was reported under the exposure of IMI, CZ and MN (Fig -2.5). Kiss2 mRNA expression was found to be significantly up-regulated (*p<0.05). The order of increase was maximum (Fig-2.5) in case of micronutrient mixture (MN) followed by insecticide (IMI) and fungicide (CZ) in hypothalamic region. On the other hand, the expression pattern of kiss-I revealed down-regulation of its transcript (Fig-2.5) on exposure of all the agrochemicals, however the significance (*p<0.05) was noted in case of insecticide (IMI), micronutrient mixture (MN) and Herbicide (PE). Further, western blotting analysis was performed for *Kiss 1* and *Kiss 2* (Fig- 2.6), where significant down regulation of *Kiss 1* expression was found on exposure of all the agrochemicals. Correspondingly, *Kiss 2* expression was also attributed to be up regulated under the exposure of MN, IMI and CZ (*p<0.05). PKC gene expression was also studied in the brain to have an insight into a probable mechanistic signaling pathway, which was found to be upregulated in response to MN (*p<0.05), IMI and CZ exposure, where as PE exposure resulted a down regulation of its transcript (Fig-2.10).

*T*he expression pattern of *GtH-Ir* was studied in ovary and testis, under all the exposure the ovary and testis exhibited an up regulation in *GtH-Ir* (Fig-2.7), however, it was significant (*p<0.05) on exposure of IMI and CZ. Analogous results were obtained for *GtH-IIr*, where an up regulation was observed in ovary and testis for all the class of agrochemicals (Fig-2.7), but the significant change (*p<0.05) was noted under the exposure of MN and IMI.

ER-I & ER-II: The expression of estrogen receptor (*ER-I & ER-II*) was studied in brain, ovary and testis to witness the change in the tissue level gene expression (Fig-2.8).ER-I and ER- II illustrated a significant up regulation (*p<0.05) on exposure of IMI, MN and CZ in brain, ovary and testis. However, PE exposure resulted in an in significant down regulation of ER I expression in brain, ovary and testis. Nonetheless, this pattern of expression was not true for ER-II, which resulted in a significant (*p<0.05) up regulation under the influence PE in ovary, with

an in significant up regulation in testis and brain. To understand the probable mechanism by which the transcript are operative MAPK was also studied. Expression of MAPK resulted into an insignificant decrease on exposure to agrochemicals (Fig2.10).

Gene expression of both the isoforms of AR (AR I and AR II) reflected distinct alterations on exposure of all agrochemicals. However, at individual level a significant (*$p<0.05$) increase in AR I and AR II was observed on exposure of CZ in brain, ovary and testis with a parallel increase on exposure of IMI only in ovary (Fig 2.9).

The results were extended by linear regression analysis of data which showed that the dependency of GnRH-I, GtH-Ir, GtH-IIr, ER-I, ER-II, AR-I and AR-II was found to be strongly correlated with the kiss 2 expression under the exposure of all the agrochemicals (table-2.9-2.12). IMI exposure resulted in significant association of AR-II with kiss-2 gene expression (fig-2.13-2.18, R^2=0.93,*$p<0.05$), similarly PE exposed groups also showed significant association of GnRH-I, GtH-Ir, ER-I, AR-I and AR-II with kiss 2 mRNA expression (R^2 >0.9,*$p<0.05$). Fishes exposed to CZ showed significant dependency of GnRH-I, GtH-IIr and AR-II on Kiss-II with a R^2>0.93,*$p<0.05$, while MN groups also showed dependency of GnRH-I, GtH-Ir, AR-II on kiss 2 genes (R^2>0.92,*$p<0.05$).

HPI:

The candidate gene studied for HPI axis was Glucocorticoid receptor (GR) in vital tissues (liver, brain, kidney, gills, thyroid, ovary and testis). Of all the agrochemicals, only IMI exposure resulted in a significant (*$p<0.05$) down regulation of GR in all the tissues along with a significant (*$p<0.05$) down regulation in testis and ovaries of fish exposed to CZ. There was an alteration (Fig-2.13, Table-2.7): in the expression in other tissues also but it was non-significant on exposure of PE and MN.

HPT:

In case of HPT axis, TSH-βr was studied in thyroid tissue where a significant (*$p<0.05$) up regulation was noted under the exposure of PE, while IMI showed up regulation of receptor expression but was non-significant (Fig-2.11, Table-2.7). In contrast, exposure of CZ and MN resulted in a in significant down regulation of TSH-βr. ($p>0.05$).

Gene	Control	CZ	IMI	MN	PE
GnRH-I	0.909±0.02	0.916±0.003	0.974±0.02	1.067±0.08	0.684±0.04
Kiss 2	0.683±0.04	0.913±0.006	1.044±0.07	1.178±0.01	0.827±0.03
Kiss 1	0.746±0.005	0.592±0.08	0.544±0.04	0.438±0.09	0.386±0.05
ER-I	0.374±0.01	0.452±0.02	0.638±0.05	0.461±0.04	0.312±0.05
ER-II	0.096±0.007	0.226±0.03	0.150±0.09	0.108±0.07	0.148±0.07
AR-I	0.621±0.09	0.993±0.02	0.710±0.08	0.674±0.03	0.648±0.04
AR-II	0.651±0.003	1.032±0.6	0.710±0.08	0.681±0.05	0.602±0.07
PKC	1.128±0.02	1.237±0.2	1.663±0.5	1.640±0.06	1.022±0.28
MAPK (ovary)	0.47±0.02	0.22605±0.01	0.3132±0.07	0.3844±0.03	0.2016±0.09

Table 2.2: Depicts the mean± SD values of HPG axis candidate genes of *O. mossambicus* exposed to agrochemicals.

Gene	CZ	IMI	MN	PE
GnRH-I	0.09	0.1	0.07	0.03
Kiss 2	**0.01***	**0.03***	**0.02***	0.75
Kiss 1	**0.04***	**0.023***	**0.032***	**0.013***
Brain ER-I	0.78	**0.025***	0.57	0.489
Brain ER-II	**0.012***	**0.021***	0.09	0.068
Brain AR-I	**0.029***	0.72	0.61	0.131
Brain AR-II	**0.034***	0.612	0.573	0.167
PKC	**0.038***	**0.031***	0.54	0.23
MAPK2	0.341	0.421	0.213	0.345

Table 2.3: Depicts the Post hoc Dunnette's multiple comparison test values of HPG axis candidate genes of *O. mossambicus* exposed to agrochemicals.(*) denotes *p<0.05,**p<0.01,**p<0.001

Genes	Control		CZ		IMI		MN		PE	
	Ovary	Testis	Ovary	Testis	Ovary	Testis	Ovary	Testis	Ovary	Testis
GtH-Ir	0.347 ±0.02	0.35± 0.07	0.749± 0.09	0.85± 0.04	0.9819 ±0.056	0.9985 ±0.079	0.49± 0.019	0.474 ±0.03	0.43± 0.06	0.455 ±0.03
GtH-IIr	0.99± 0.03	1.033 ±0.08	1.15± 0.09	1.17± 0.09	1.529± 0.02	1.575± 0.043	1.631 ±0.05	1.649 ±0.07	1.29± 0.04	1.31± 0.09
ER-I	0.345 ±0.03	0.324 ±0.05	0.439± 0.06	0.415 ±0.02	0.681± 0.07	0.3769 ±0.06	0.499 ±0.08	0.372 ±0.02	0.249 ±0.01	0.229 ±0.01
ER-II	0.091± 0.001	0.122 ±0.04	0.212± 0.03	0.249 ±0.07	0.083± 0.08	0.158± 0.04	0.107 ±0.02	0.151 ±0.06	0.180 ±0.08	0.186 ±0.08
AR-I	0.55 ±0.04	0.58 ±0.03	0.96 ±0.05	0.98± 0.05	0.877 ±0.06	0.697 ±0.05	0.635 ±0.07	0.620 ±0.03	0.589 ±0.02	0.549 ±0.04
AR-II	0.673 ±0.05	0.561 ±0.04	1.088 ±0.07	1.057 ±0.07	0.747 ±0.06	0.889 ±0.06	0.715 ±0.05	0.703 ±0.05	0.678 ±0.05	0.649 ±0.05

Table 2.4: Depicts the mean± SD values of HPG axis candidate genes in ovary and testes of *O. mossambicus* exposed to agrochemicals.

Genes	CZ		IMI		MN		PE	
	Ovary	Testis	Ovary	Testis	Ovary	Testis	Ovary	Testis
GtH-Ir	**0.006****	**0.005****	**0.004****	**0.004****	0.091	0.087	0.089	0.075
GtH-IIr	0.657	0.592	0.341	0.243	0.048	0.031	0.678	0.654
ER-I	0.98	0.78	**0.021***	**0.038***	**0.029***	**0.031***	0.09	0.092
ER-II	**0.023***	**0.031***	0.23	0.34	0.56	0.87	**0.034***	**0.026***
AR-I	**0.022***	**0.028***	0.031	0.038	0.78	0.86	0.65	0.64
AR-II	**0.012***	**0.026***	0.09	0.042	0.231	0.172	0.233	0.322
MAPK	0.341	0.421	0.213	0.345	0.567	0.67	0.78	0.89

Table 2.5: Depicts the Post hoc Dunnette's multiple comparison test values of HPG axis candidate genes of *O. mossambicus* exposed to agrochemicals.(*) denotes *p<0.05,**p<0.01,**p<0.001

Gene	Control	CZ	IMI	MN	PE
Kiss 2	3.98±0.04	4.13±0.42	5.12±0.04	8.10±0.09	2.12±0.02
Kiss 1	7.76±0.03	3.83±0.04	3.41±0.04	2.37±0.01	1.76±0.08

Table 2.6: Depicts the western blot mean± SD values of kiss2 and kiss 1 in brain of *O. mossambicus* exposed to agrochemicals.

Tissues	Control	CZ	IMI	MN	PE	Genes
Brain	1.33±0.05	1.36±0.06	1.04±0.04	1.31±0.07	1.19±0.08	
Liver	1.28±0.08	1.36±0.09	0.92±0.06	1.24±0.05	1.32±0.04	
Gills	1.30±0.02	1.30±0.08	0.69±0.02	1.36±0.05	1.08±0.03	
Kidney	1.32±0.02	1.09±0.04	0.80±0.01	1.31±0.04	1.30±0.03	**GR**
Thyroid	1.38±0.03	1.25±0.05	0.71±0.03	1.24±0.03	1.22±0.04	
Ovary	1.53±0.02	0.62±0.05	0.48±0.03	1.24±0.03	1.17±0.04	
Testis	1.36±0.04	0.57±0.03	0.43±0.06	1.20±0.06	1.13±0.08	
Thyroid	0.57±0.04	0.46±0.06	0.85±0.03	0.53±0.06	0.60±0.03	**TSH-βr**

Table 2.7: Depicts the mean± SD values of GR and TSH-βr in vital tissues of *O. mossambicus* exposed to agrochemicals.

Tissues	CZ	IMI	MN	PE	Genes
Brain	0.91	**0.034***	0.76	0.54	
Liver	0.23	**0.029***	0.45	0.65	
Gills	0.45	**0.031***	0.42	0.188	
Kidney	0.097	**0.039***	0.24	0.28	**GR**
Thyroid	0.42	**0.035***	0.19	0.47	
Ovary	**0.006**	**0.003***	0.09	0.07	
Testis	**0.005**	**0.002***	0.06	0.09	
Thyroid	0.78	**0.031***	0.89	0.97	**TSH-βr**

Table 2.8: Depicts the Post hoc Dunnette's multiple comparison test values of GR and TSH- βr axis candidate genes of *O. mossambicus* exposed to agrochemicals.(*) denotes *p<0.05,**p<0.01,**p<0.001

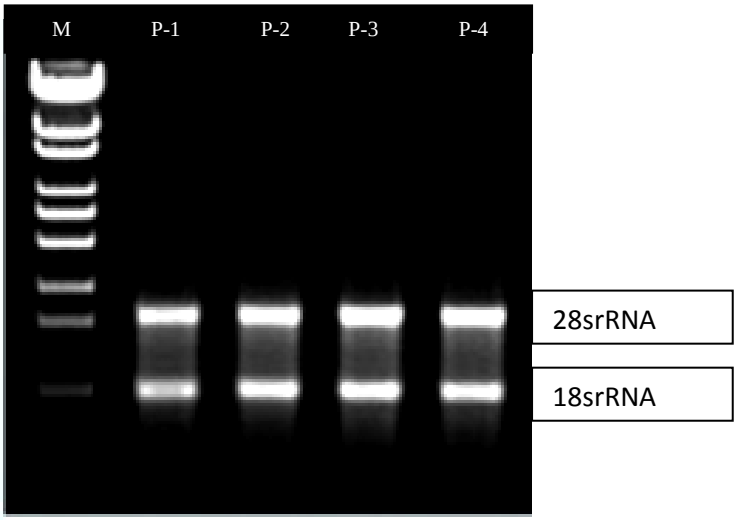

Figure 2.1: 1.5% Agarose gel image of total RNA isolated. M-Marker, P1-P4-Samples

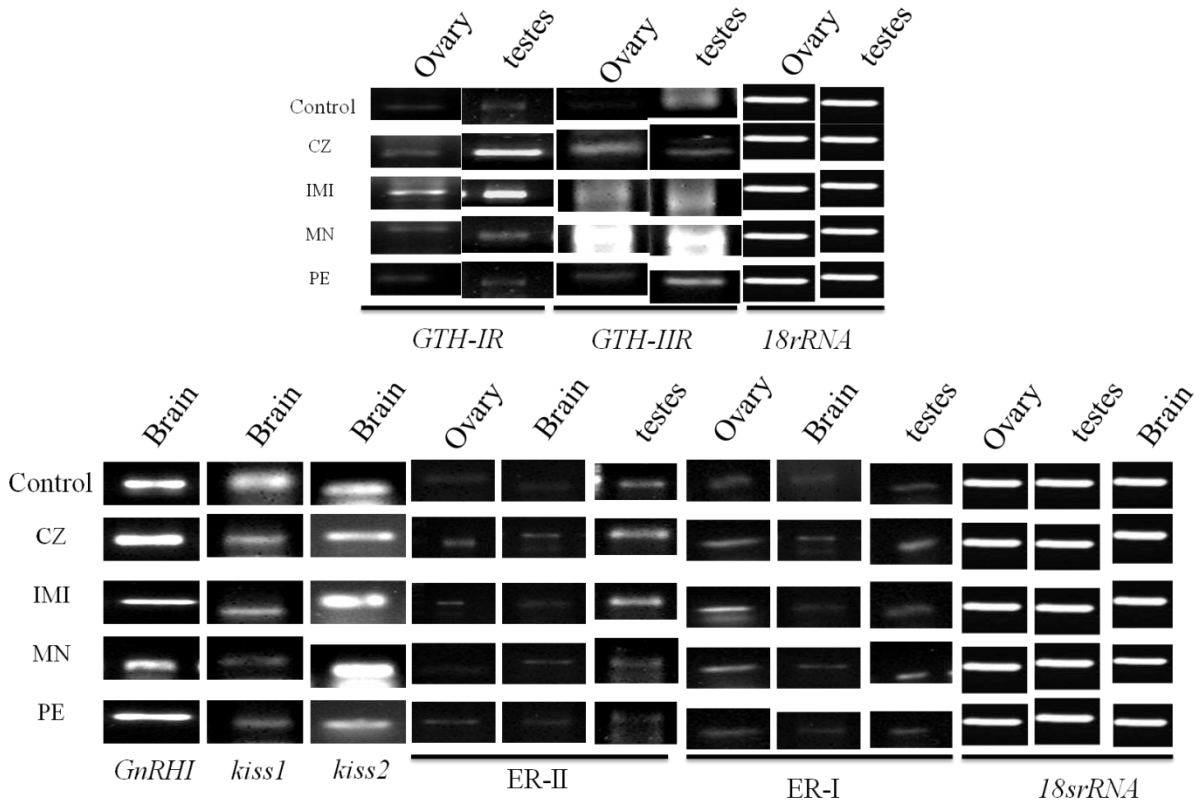

Figure 2.2 : 2% agarose gene expression images of Kisspeptin 1 & 2, GnRH I-Gonadotropin Releasing Hormone, GtHIr & GtHIIr-Gonadotropins Hormone Receptor, ERI& ERII Estrogen receptor. C-Control, CZ-Curzate, IMI-Imidacloprid, MN-Micronutrient Mixture, PE-Pyzosulphuron ethyl. (18srRNA was taken as the internal control).

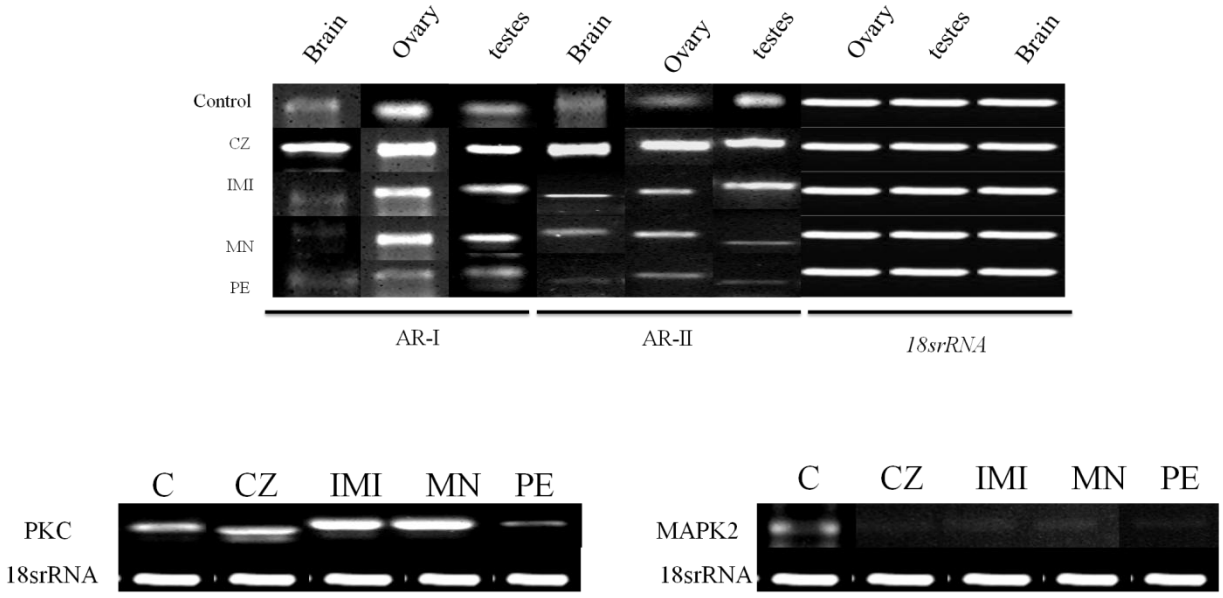

Figure 2.3 : 2% agarose gene expression images of ARI and II Androgen receptor in brain ovary and testis, PKC in hypothalamic region and MAPK2 in ovary. C-Control, CZ-Curzate, IMI-Imidacloprid, MN-Micronutrient Mixture, PE-Pyzosulphuron ethyl. (18srRNA was taken as the internal control).

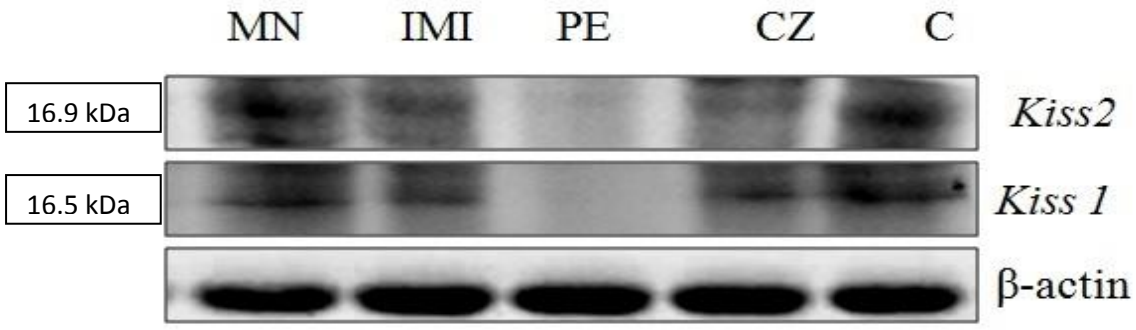

Figure 2.4: Western blot analysis of kisspeptin 1 and kisspeptin 2. β-actin was taken as internal control for densitometric analysis.

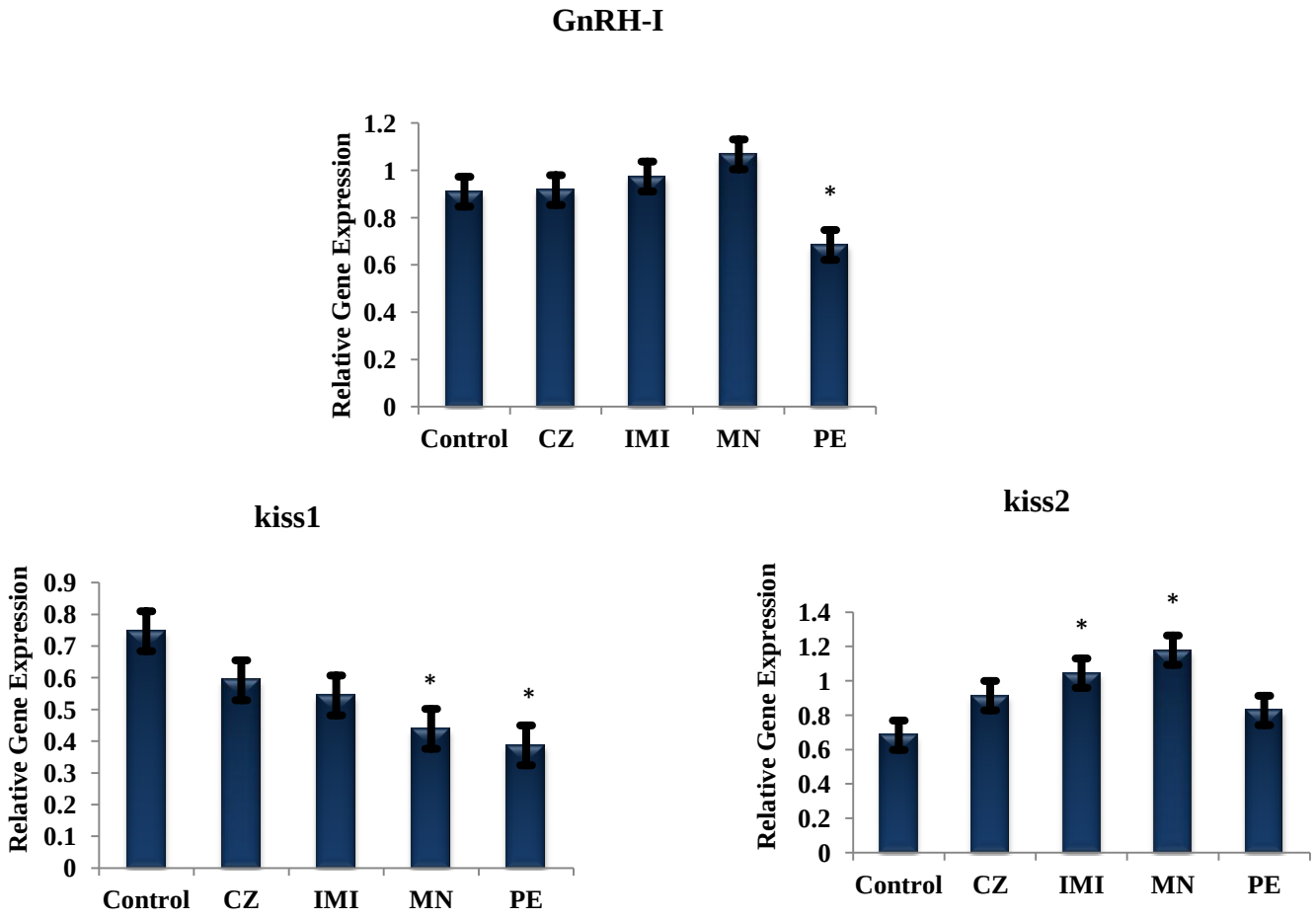

Figure 2.5: Relative gene expression pattern of *GnRH-I, kiss1, kiss2* in brain hypothalamic region. (*) denotes level of significance at *p<0.05, **p<0.01,***p<0.001.

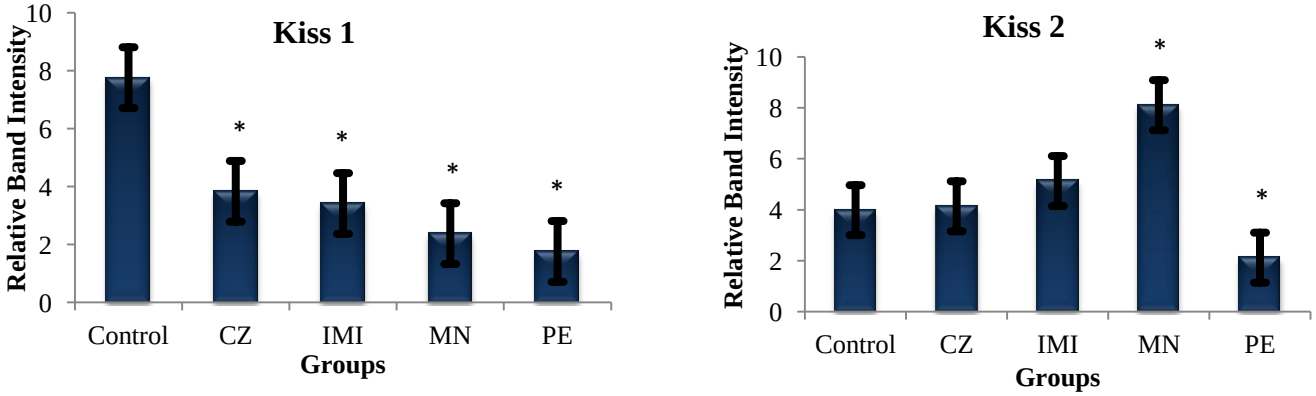

Figure 2.6: Relative band density of *Kiss 1* and *Kiss 2* of 4 groups compared to control. (*) denotes level of significance at *p<0.05, **P<0.01, ***P<0.001

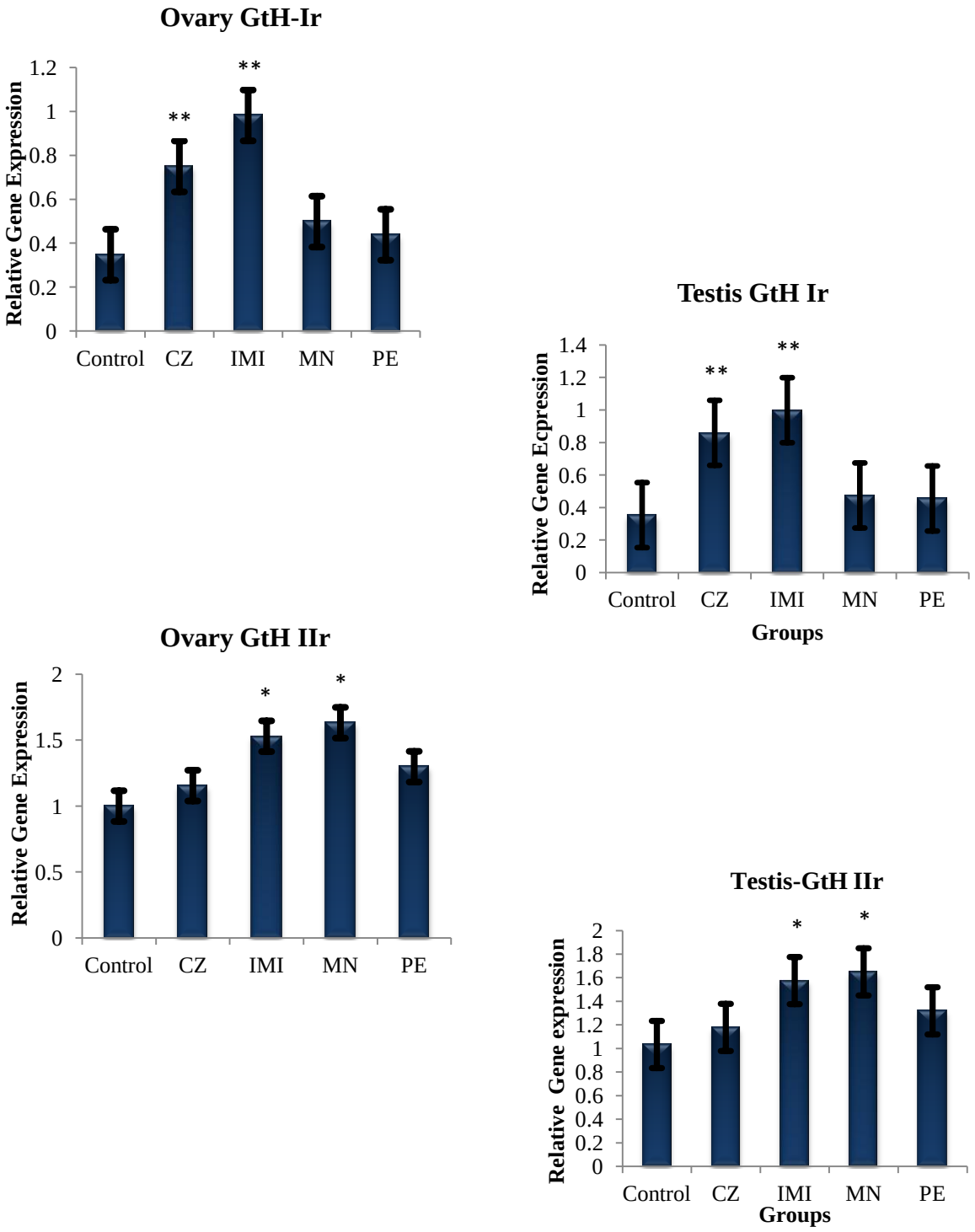

Figure 2.7: Relative gene expression pattern of GtH-Ir, GtH-IIr in ovary and testes. (*) denotes level of significance at *p<0.05,**p<0.01,***p<0.001.

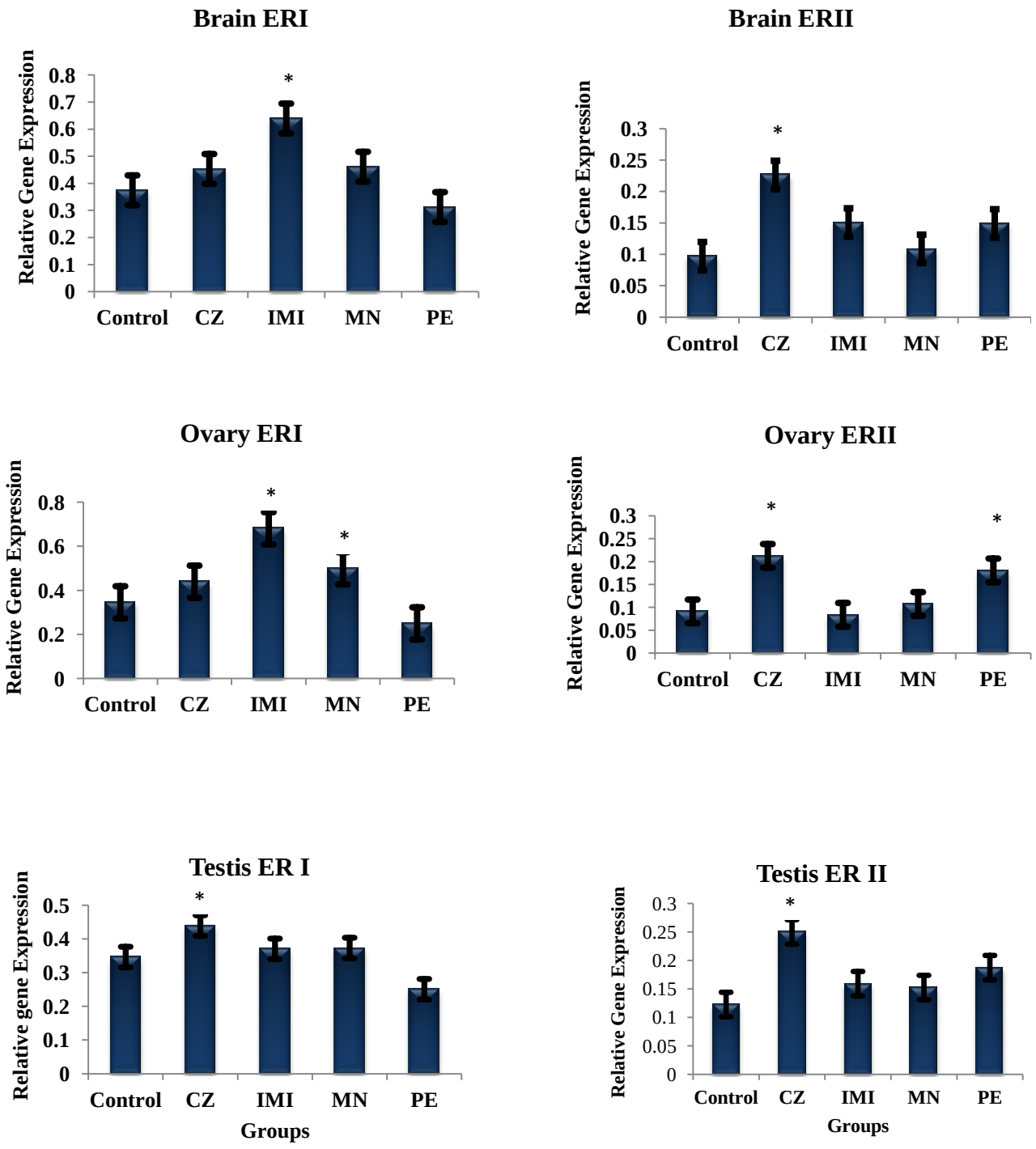

Figure 2.8: Relative gene expression pattern of ER-I, ER-II in Brain, ovary and testes. (*) denotes level of significance at *p<0.05,**p<0.01,***p<0.001.

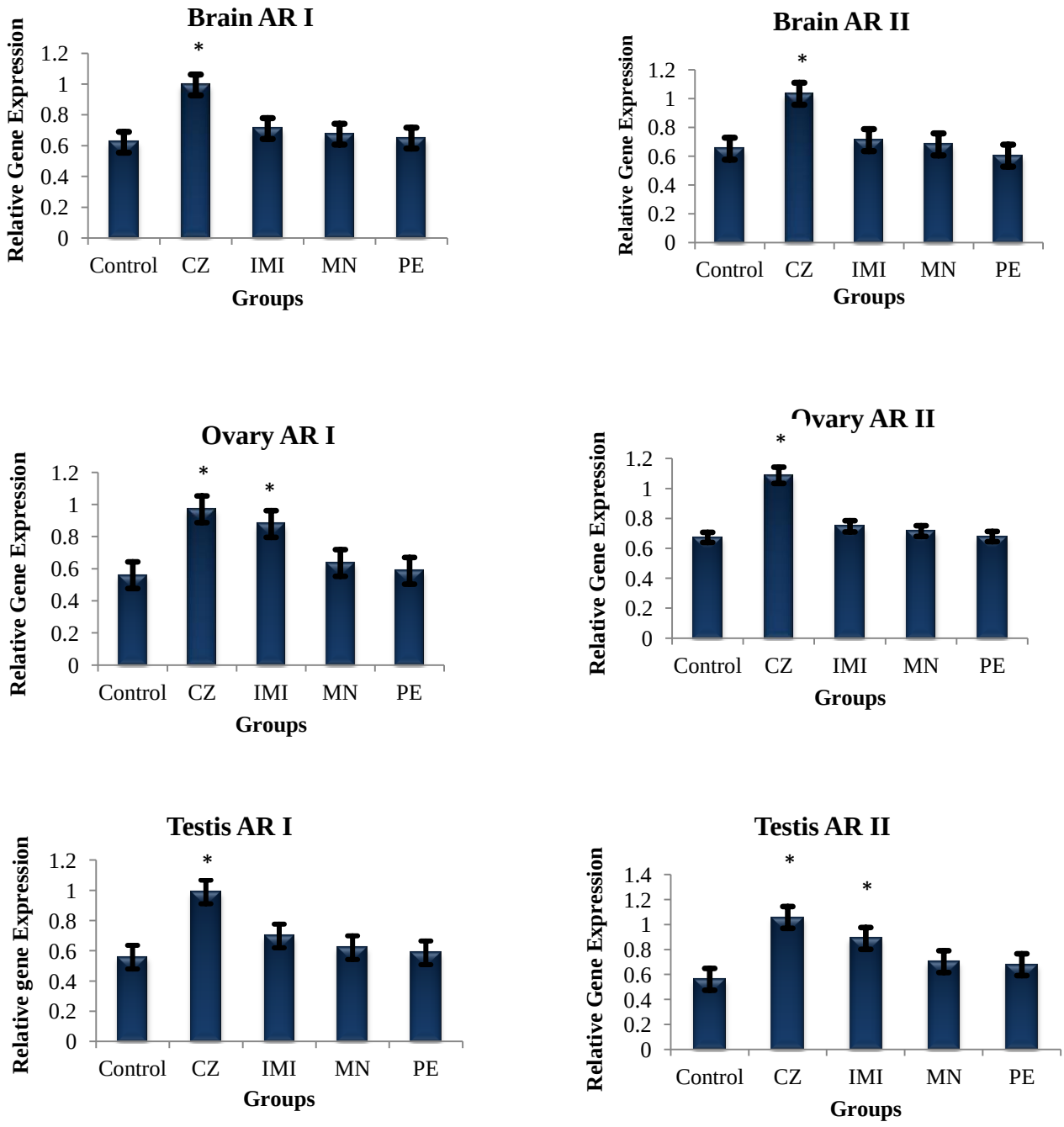

Figure 2.9: Relative gene expression pattern of AR-I, AR-II in Brain, ovary and testes. (*) denotes level of significance at *p<0.05,**p<0.01,***p<0.001.

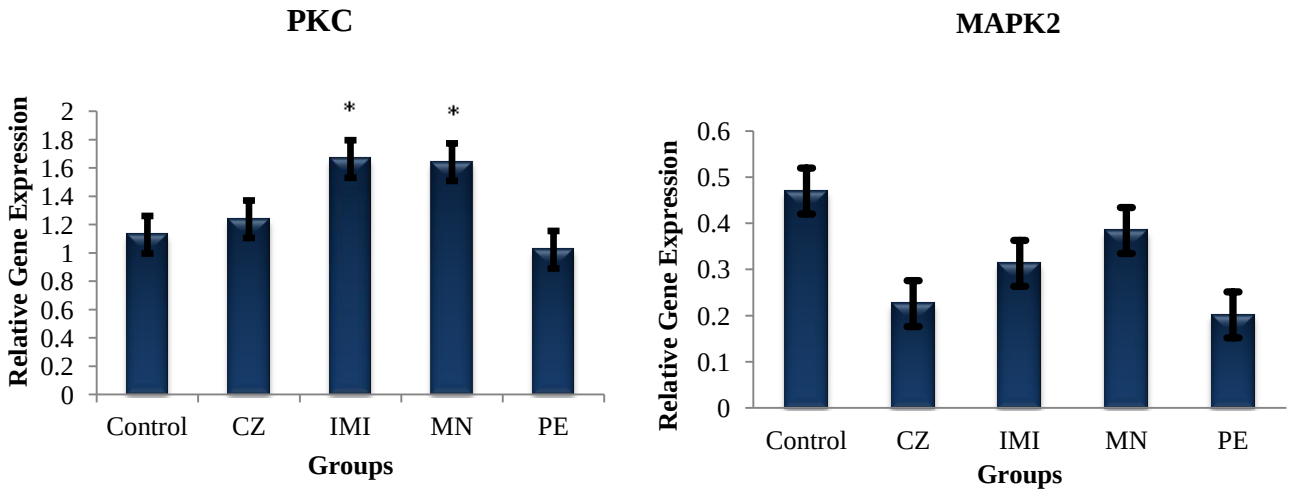

Figure 2.10: Relative gene expression pattern of PKC in brain hypothalamic region and MAPK2 in ovary. (*) denotes level of significance at *p<0.05, **p<0.01,***p<0.001.

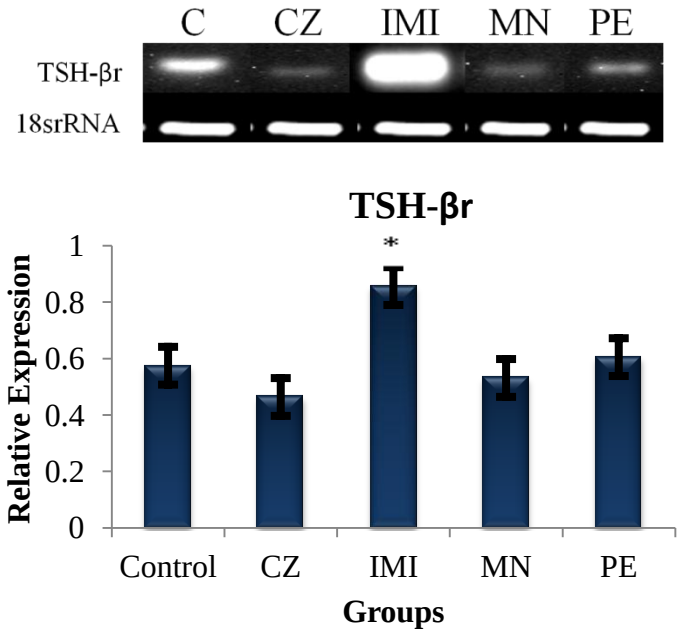

Figure 2.11: 2% agarose gel image with relative gene expression pattern of TSH-βr in thyroid gland. (*) denotes level of significance at *p<0.05,**p<0.01,***p<0.001.

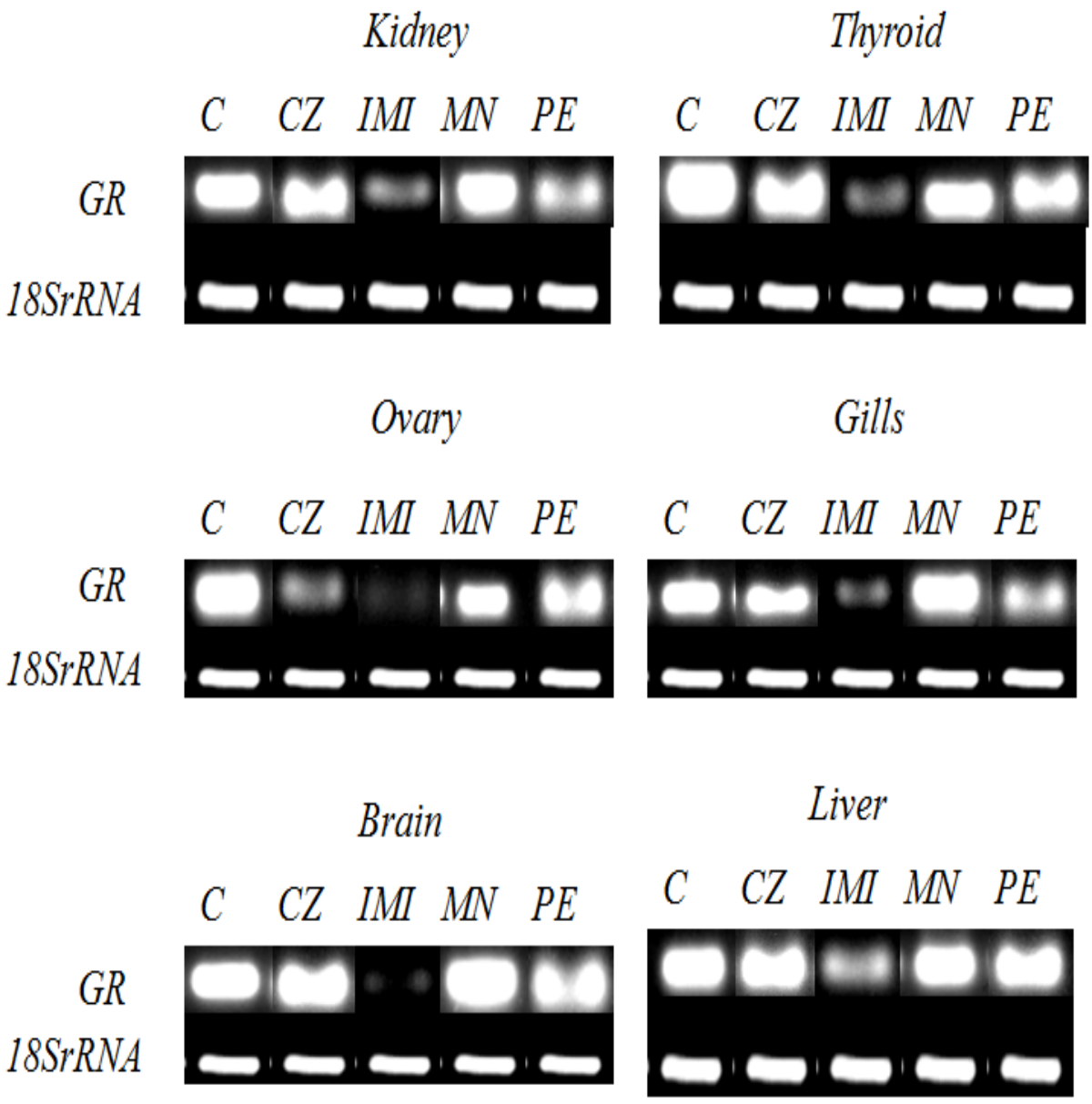

Figure 2.12: 2% Agarose Gene expression images of GR-Glucocorticoid receptor in kidney, thyroid, ovary gills, brain and liver. C-Control, CZ-Curzate, IMI-Imidacloprid, MN-Micronutrient Mixture, PE-Pyzosulphuron ethyl

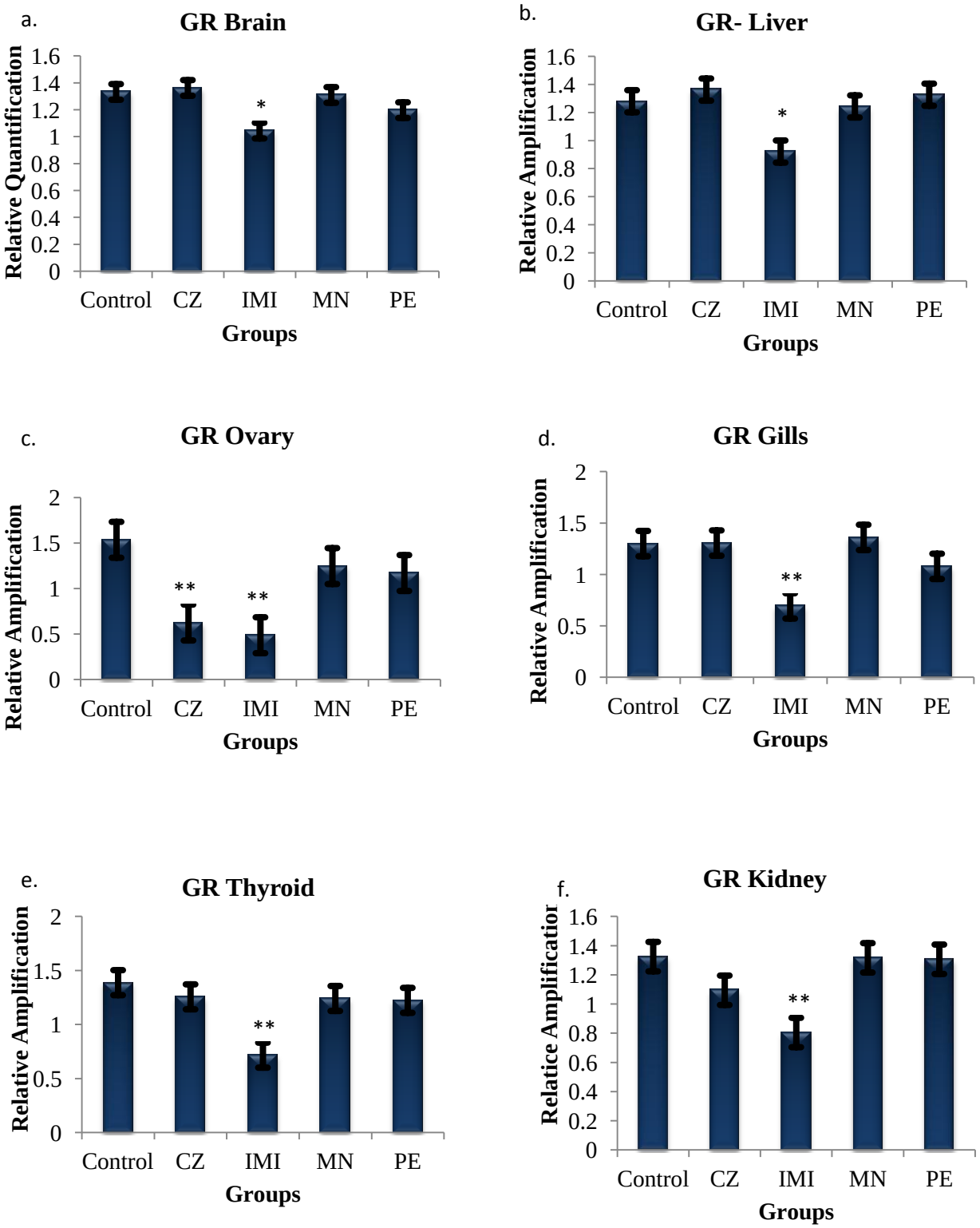

Figure 2.13(a-f): Graph showing relative expression pattern of the Glucocorticoid receptor (GR) in four different groups of *O.mossambicus* exposed to agrochemicals.(*) denotes the significance of *p<0.05, **p<0.01, ***p<0.001.

	R^2 Value	*Coefficients*	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
GnRH-I vs. Kiss 2							
GnRH Intercept	0.93310	0.155642	0.091988	1.69198	0.151434	-0.08082	0.392105
Kiss 2		0.83324	0.099775	8.351212	0.000403	0.576761	1.089719
GtH-Ir vs. Kiss 2							
GtH-Ir Intercept	0.838205	0.043201	0.138029	0.312986	0.766925	-0.31161	0.398017
Kiss 2		0.761969	0.149713	5.089524	0.003804	0.377119	1.146819
GtH-IIr vs. Kiss 2							
GtH-IIr Intercept	0.93297	0.023167	0.133625	0.173374	0.869158	-0.32033	0.366661
Kiss 2		1.209094	0.144936	8.342254	0.000405	0.836524	1.581664
ER-I vs. Kiss 2							
ER-I Intercept	0.87626	-0.83004	0.212705	-3.90232	0.011383	-1.37682	-0.28327
Kiss 2		1.372849	0.23071	5.950542	0.001916	0.77979	1.965907
ER-II vs. Kiss 2							
ER-II Intercept	0.79843	-1.11553	0.298591	-3.73598	0.013487	-1.88308	-0.34798
Kiss 2		1.441315	0.323866	4.450343	0.0067	0.608791	2.273839
AR-I vs. Kiss 2							
AR-I Intercept	0.936347	-0.70164	0.191426	-3.66535	0.014514	-1.19372	-0.20957
Kiss 2		1.780666	0.207629	8.57618	0.000355	1.246938	2.314394
AR-II vs. Kiss 2							
AR-II Intercept	0.885627	-3.61024	0.753796	-4.78941	0.004929	-5.54793	-1.67255
Kiss 2		5.087342	0.817603	6.222268	0.001568	2.985628	7.189056

Table 2.9: Regression Analysis of kisspeptin 2 with Candidate HPG genes of *O.mossambicus* exposed to CZ.

	R^2 Value	*Coefficients*	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
GnRH-I vs. Kiss 2							
GnRH Intercept	0.925813	0.868245	0.023253	37.33938	2.46E-08	0.811348	0.925143
Kiss 2		0.166009	0.019185	8.653131	0.000131	0.119065	0.212953
GtH-Ir vs. Kiss 2							
GtH-Ir Intercept	0.755845	-0.99891	0.330116	-3.02594	0.023219	-1.80668	-0.19115
Kiss 2		1.173841	0.272364	4.309821	0.005038	0.50739	1.840292
GtH-IIr vs. Kiss 2							
GtH-IIr Intercept	0.8531	-0.72038	0.390512	-1.84471	0.114628	-1.67593	0.235167
Kiss 2		1.901879	0.322194	5.902902	0.001051	1.113499	2.690258
ER-I vs. Kiss 2							
ER-I Intercept	0.898756	0.096279	0.056057	1.717518	0.136692	-0.04089	0.233446
Kiss 2		0.337541	0.04625	7.298148	0.000337	0.224371	0.450711
ER-II vs. Kiss 2							
ER-II Intercept	0.116428	2.044987	1.771869	1.154141	0.292333	-2.29062	6.380595
Kiss 2		-1.29986	1.46189	-0.88917	0.408146	-4.87698	2.277254
AR-I vs. Kiss 2							
AR-I Intercept	0.83218	0.362287	0.053638	6.754264	0.000514	0.231039	0.493535
Kiss 2		0.24139	0.044255	5.454592	0.001581	0.133104	0.349677
AR-II vs. Kiss 2							
AR-II Intercept	0.004001	0.776245	0.880254	0.881842	0.411793	-1.37766	2.930148
Kiss 2		-0.11275	0.726258	-0.15525	0.881715	-1.88984	1.664337

Table 2.10: Regression Analysis of kisspeptin 2 with Candidate HPG genes of *O.mossambicus* exposed to MN.

	R^2 Value	*Coefficients*	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
GnRH-I vs. Kiss 2							
GnRH Intercept	0.57654	0.594696	0.110924	5.361307	0.000677	0.338906	0.850487
Kiss 2		0.341333	0.103425	3.30031	0.010854	0.102836	0.579831
GtH-Ir vs. Kiss 2							
GtH-Ir Intercept	0.575835	0.679164	0.087184	7.790028	5.29E-05	0.478118	0.88021
Kiss 2		0.267893	0.08129	3.295542	0.010931	0.080439	0.455347
GtH-IIr vs. Kiss 2							
GtH-IIr Intercept	0.620437	1.209467	0.092307	13.10267	1.09E-06	0.996607	1.422327
Kiss 2		0.311233	0.086066	3.616198	0.00682	0.112764	0.509702
ER-I vs. Kiss 2							
ER-I Intercept	0.413331	0.408478	0.111592	3.660472	0.006397	0.151147	0.665809
Kiss 2		0.247018	0.104047	2.374092	0.044958	0.007084	0.486951
ER-II vs. Kiss 2							
ER-II Intercept	0.28273	0.172631	0.274246	0.629475	0.54659	-0.45978	0.805043
Kiss 2		0.454076	0.255705	1.77578	0.11368	-0.13558	1.043732
AR-I vs. Kiss 2							
AR-I Intercept	0.462679	0.420431	0.16719	2.514684	0.036107	0.034889	0.805972
Kiss 2		0.409145	0.155887	2.624627	0.030431	0.049669	0.768622
AR-II vs. Kiss 2							
AR-II Intercept	0.919989	0.335393	0.041887	8.007098	0.000002	0.238802	0.431985
Kiss 2		0.374575	0.039055	9.59093	0.000004	0.284514	0.464636

Table 2.11: Regression Analysis of kisspeptin 2 with Candidate HPG genes of *O.mossambicus* exposed to IMI.

	R^2 Value	*Coefficients*	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
GnRH-I vs. Kiss 2							
GnRH Intercept	0.951501	0.159917	0.04473	3.575183	0.009034	0.054148	0.265687
Kiss 2		0.619835	0.052892	11.71887	7.45E-06	0.494765	0.744904
GtH-Ir vs. Kiss 2							
GtH-Ir Intercept	0.086733	0.603628	0.166867	3.617423	0.005594	0.226149	0.981107
Kiss 2		-0.18422	0.199265	-0.92452	0.379339	-0.63499	0.266545
GtH-IIr vs. Kiss 2							
GtH-IIr Intercept	0.036451	1.408217	0.208179	6.764463	8.23E-05	0.937284	1.87915
Kiss 2		-0.14506	0.248598	-0.5835	0.573889	-0.70742	0.417311
ER-I vs. Kiss 2							
ER-I Intercept	0.088575	0.142904	0.170441	0.838435	0.423494	-0.24266	0.528468
Kiss 2		0.190349	0.203533	0.935226	0.374085	-0.27007	0.650772
ER-II vs. Kiss 2							
ER-II Intercept	0.09843	0.188733	0.164283	1.148833	0.28024	-0.1829	0.560367
Kiss 2		0.000388	0.196179	0.001978	0.998465	-0.4434	0.444175
AR-I vs. Kiss 2							
AR-I Intercept	0.226476	0.311502	0.146999	2.11908	0.063132	-0.02103	0.644036
Kiss 2		0.284951	0.175539	1.62329	0.138976	-0.11215	0.682048
AR-II vs. Kiss 2							
AR-II Intercept	0.106349	0.480469	0.164035	2.929073	0.016782	0.109397	0.851542
Kiss 2		0.202721	0.195883	1.034911	0.327716	-0.2404	0.645839

Table 2.12: Regression Analysis of kisspeptin 2 with Candidate HPG genes of *O.mossambicus* exposed to PE.

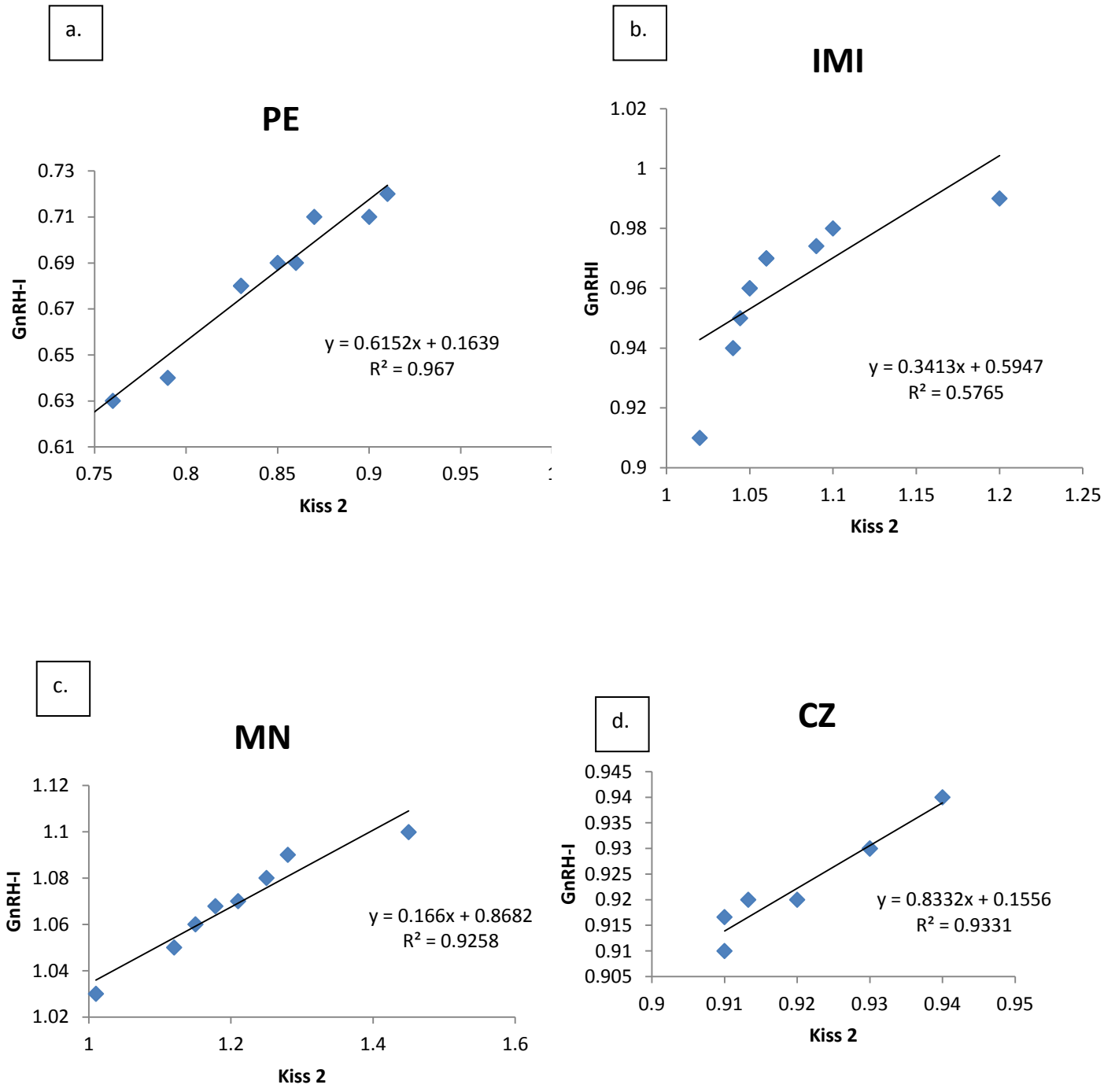

Figure 2.14(a-d): Graph showing regression analysis of kiss2 and GnRH-I of *O.mossambicus* exposed to agrochemicals.

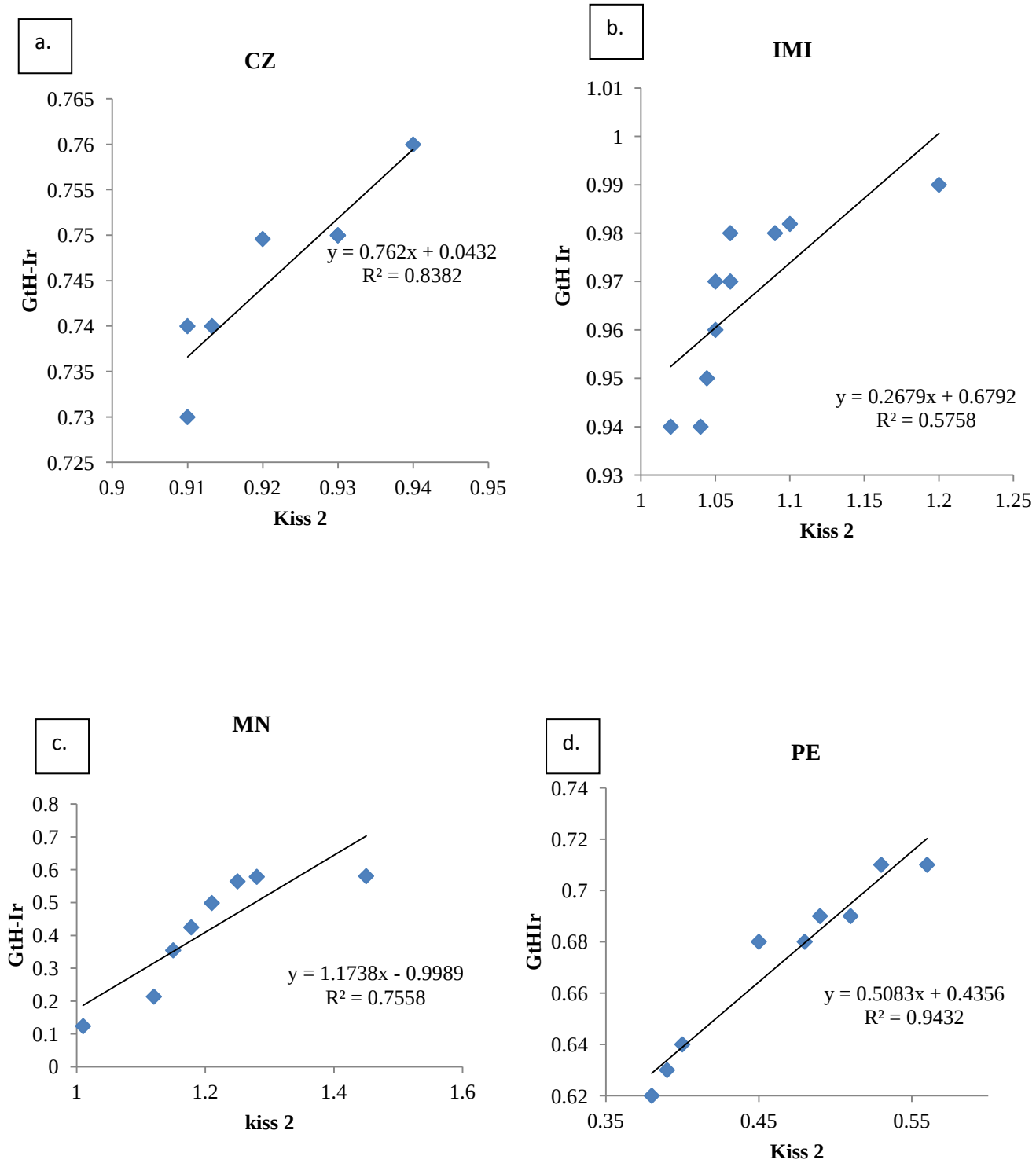

Figure 2.15(a-d): Graph showing regression analysis of kiss2 and GtH-Ir of *O.mossambicus* exposed to agrochemicals.

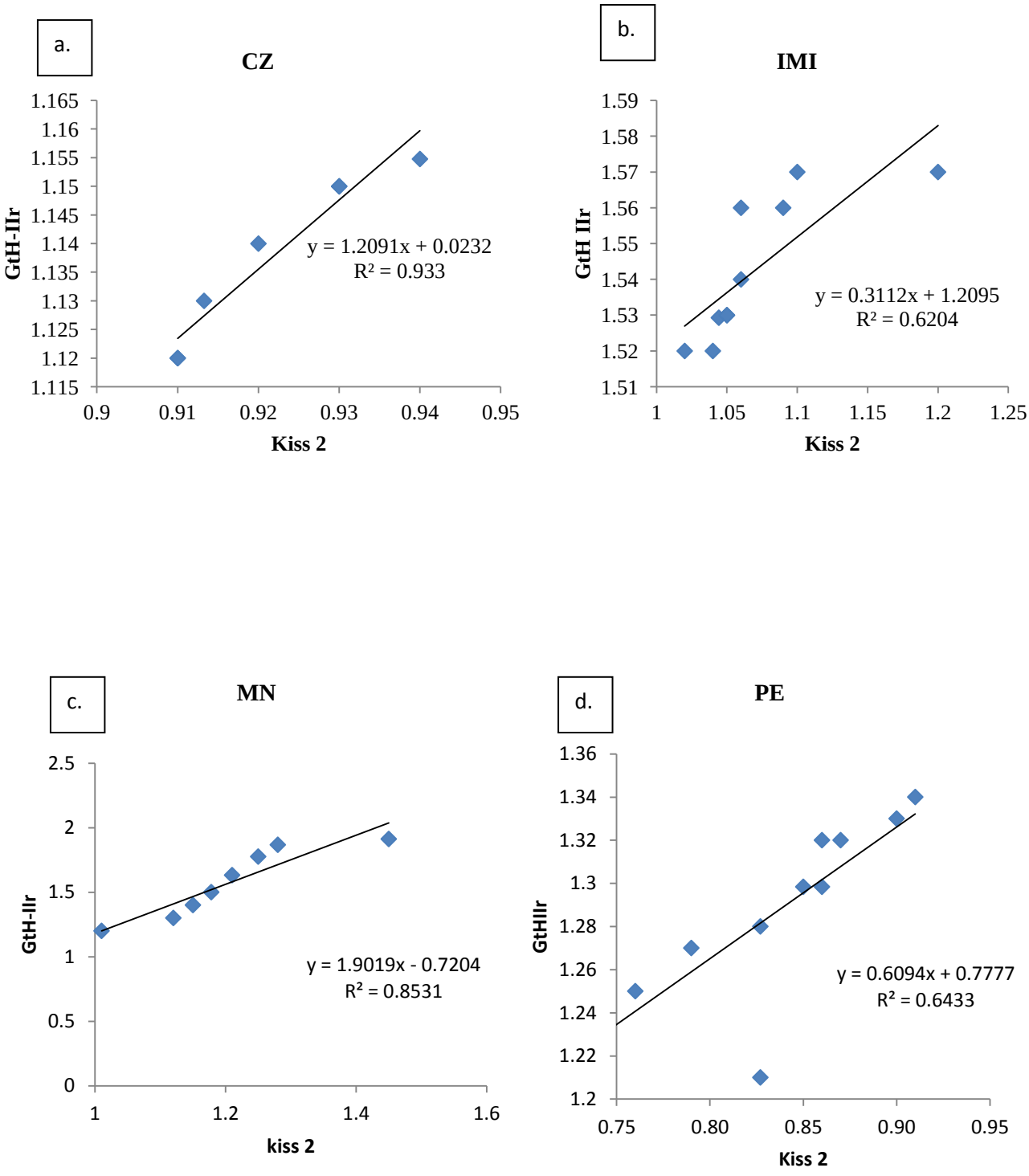

Figure 2.16(a-d): Graph showing regression analysis of kiss2 and GtH-IIr of *O.mossambicus* exposed to agrochemicals.

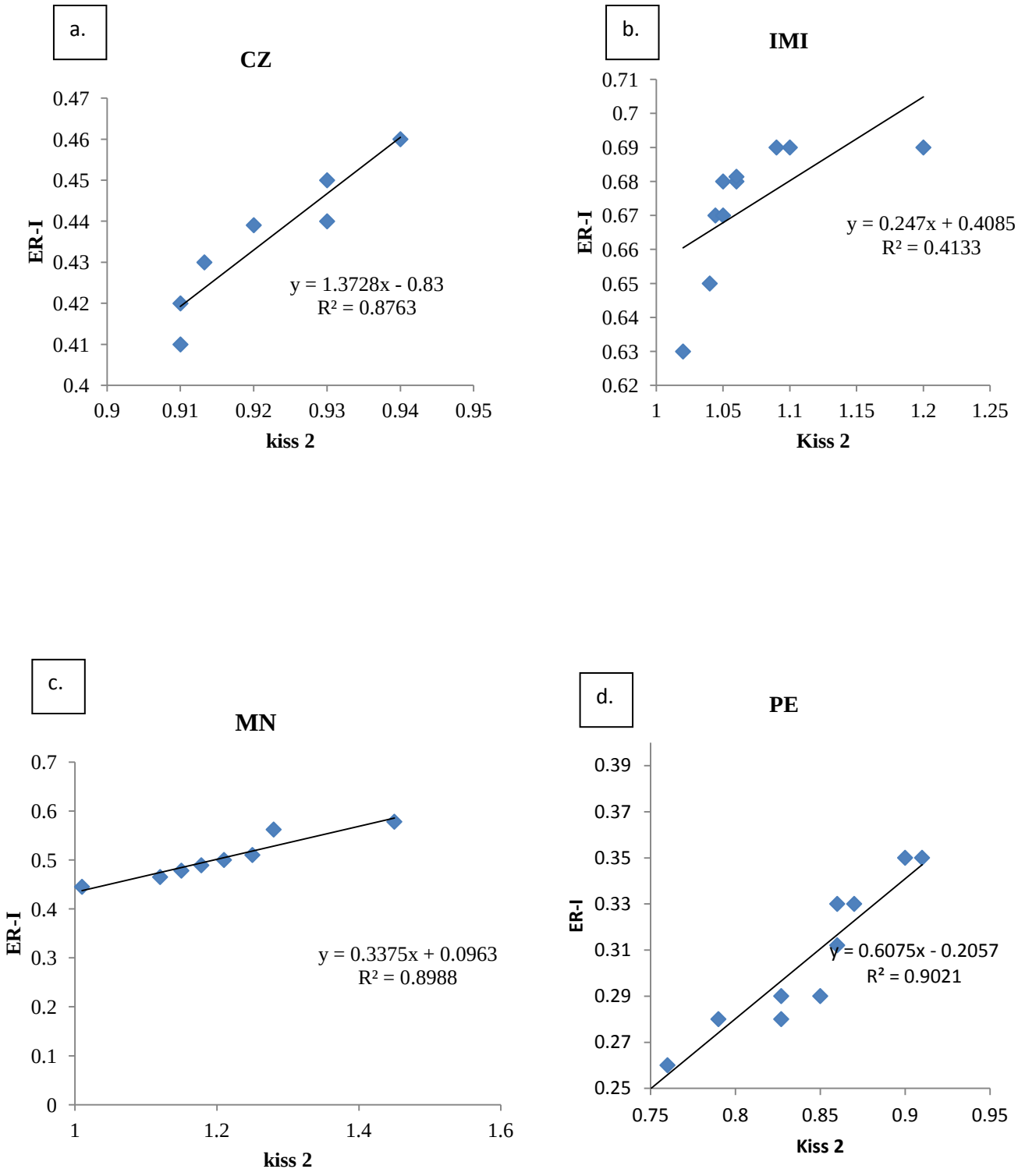

Figure 2.17(a-d): Graph showing regression analysis of kiss2 and ER-I of *O.mossambicus* exposed to agrochemicals.

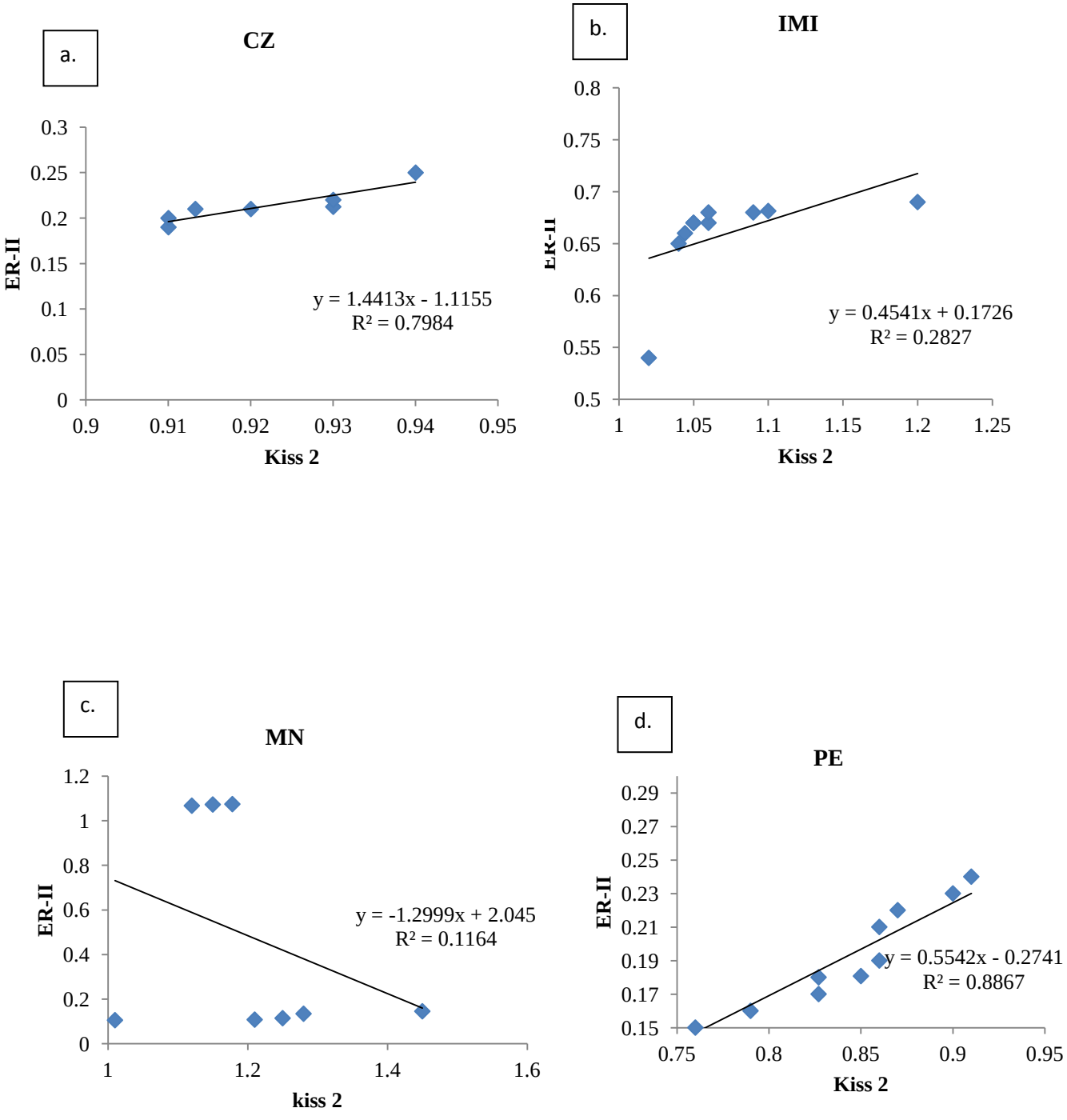

Figure 2.18(a-d): Graph showing regression analysis of kiss2 and ER-II of *O.mossambicus* exposed to agrochemicals.

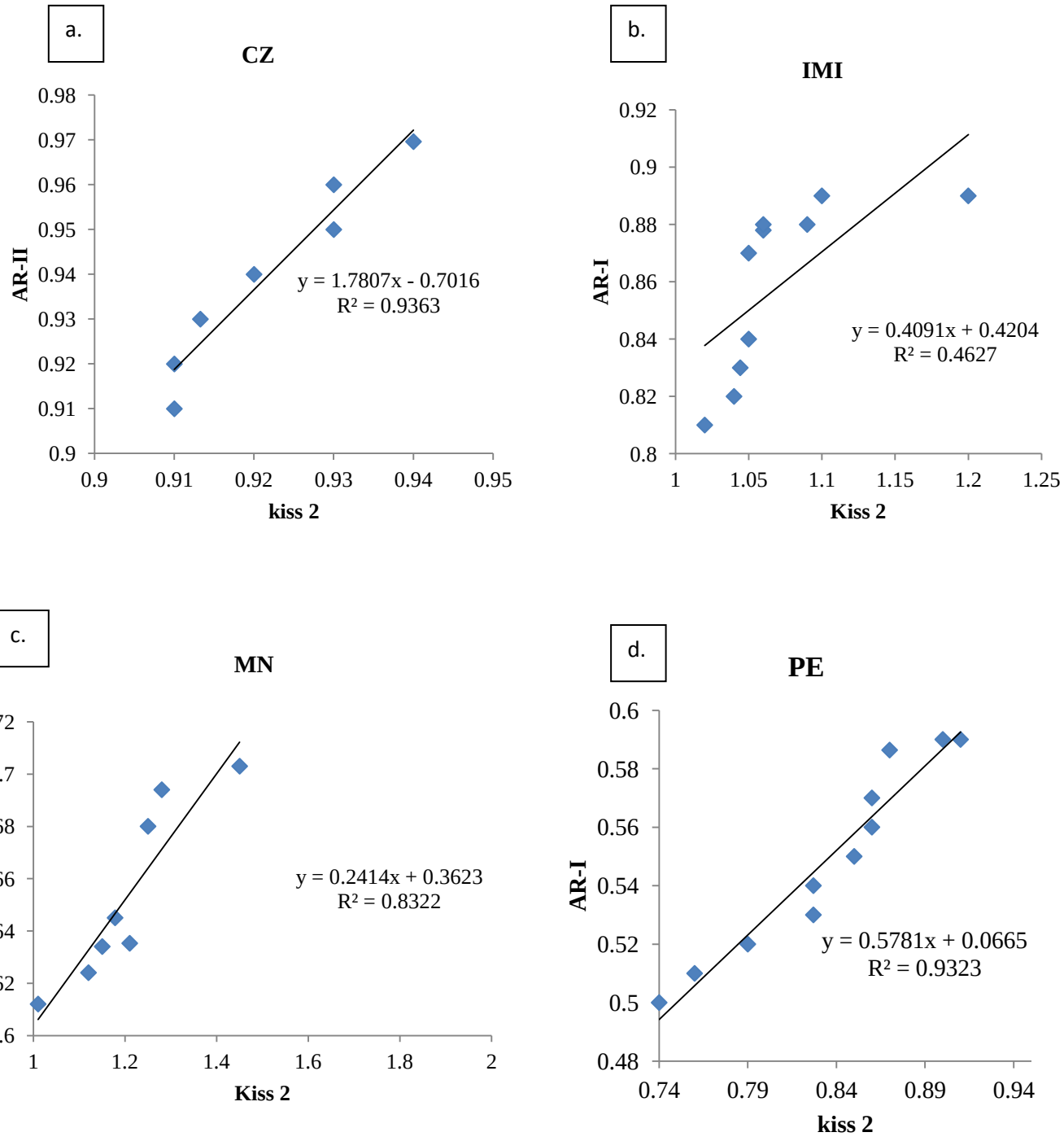

Figure 2.19(a-d): Graph showing regression analysis of kiss2 and AR-I of *O.mossambicus* exposed to agrochemicals.

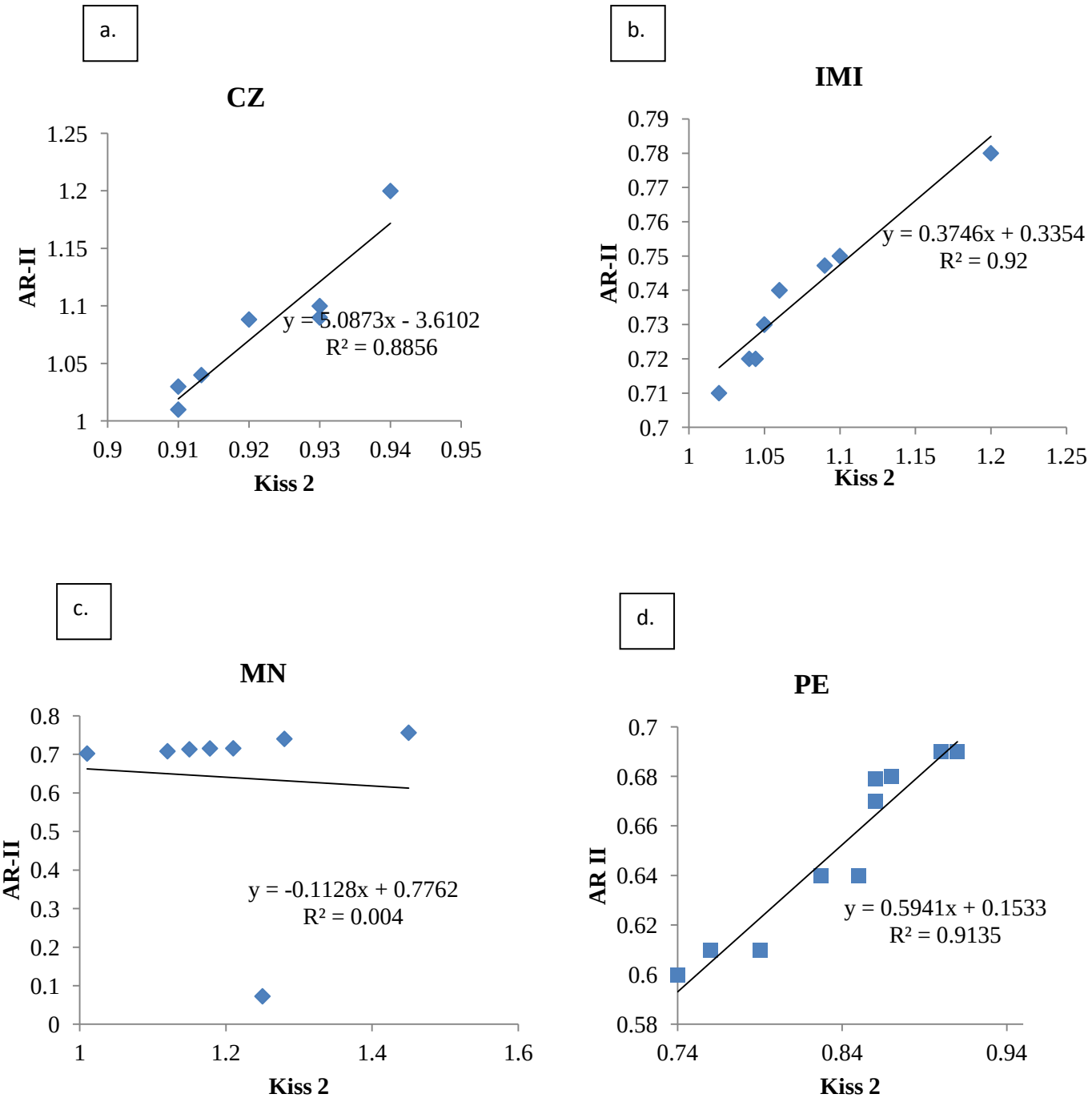

Figure 2.20(a-d): Graph showing regression analysis of kiss2 and AR-II of *O.mossambicus* exposed to agrochemicals.

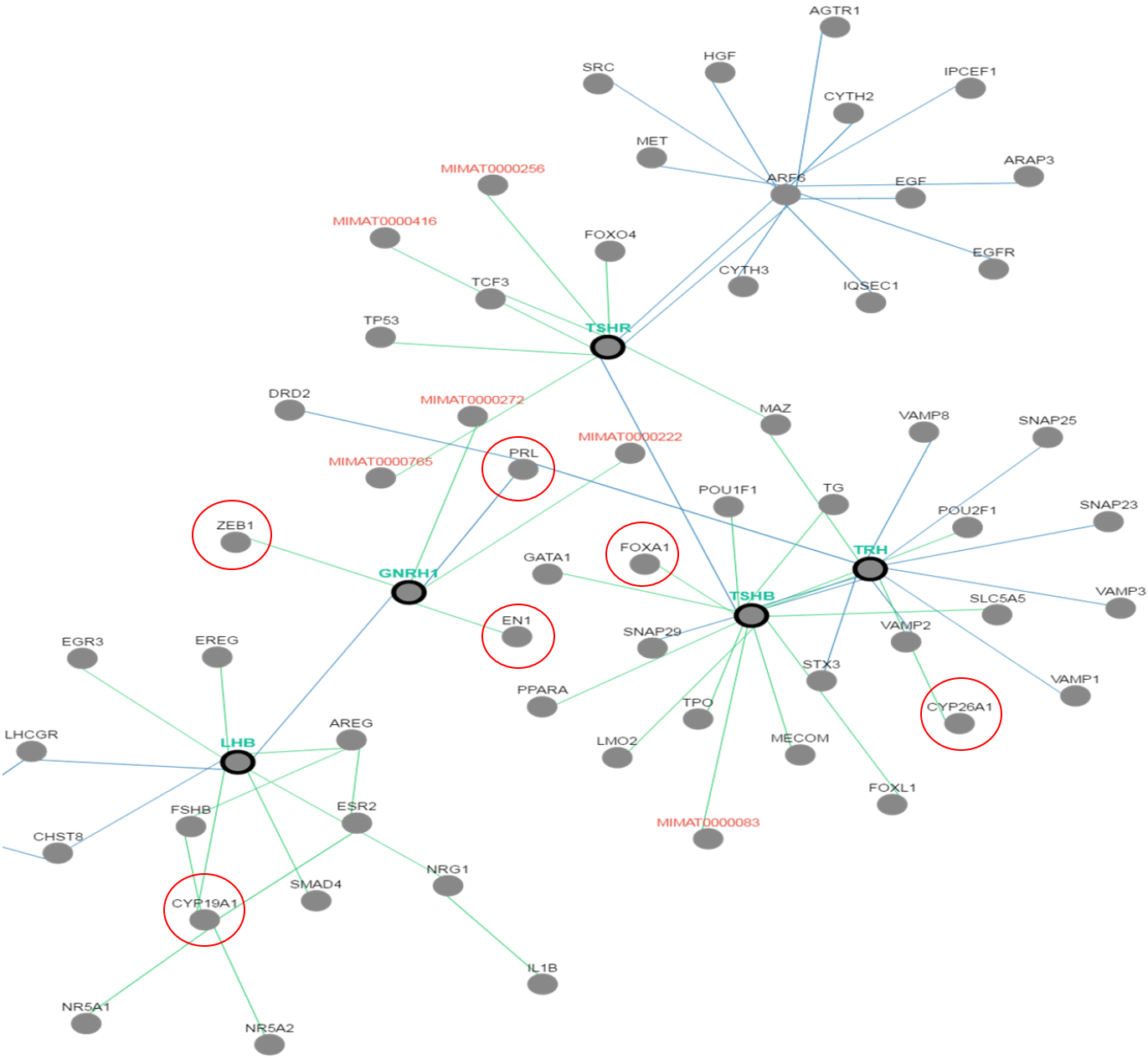

Figure 2.21: Schematic representation of GnRH-I, LHb, TSHb, TRH and TSHr genes with the associated genes. (——) Depicts control of the gene expression, (——) depicts control of the state change. Red circle genes were taken for consideration as it directly or indirectly are involved in regulation in the endocrine pathways.

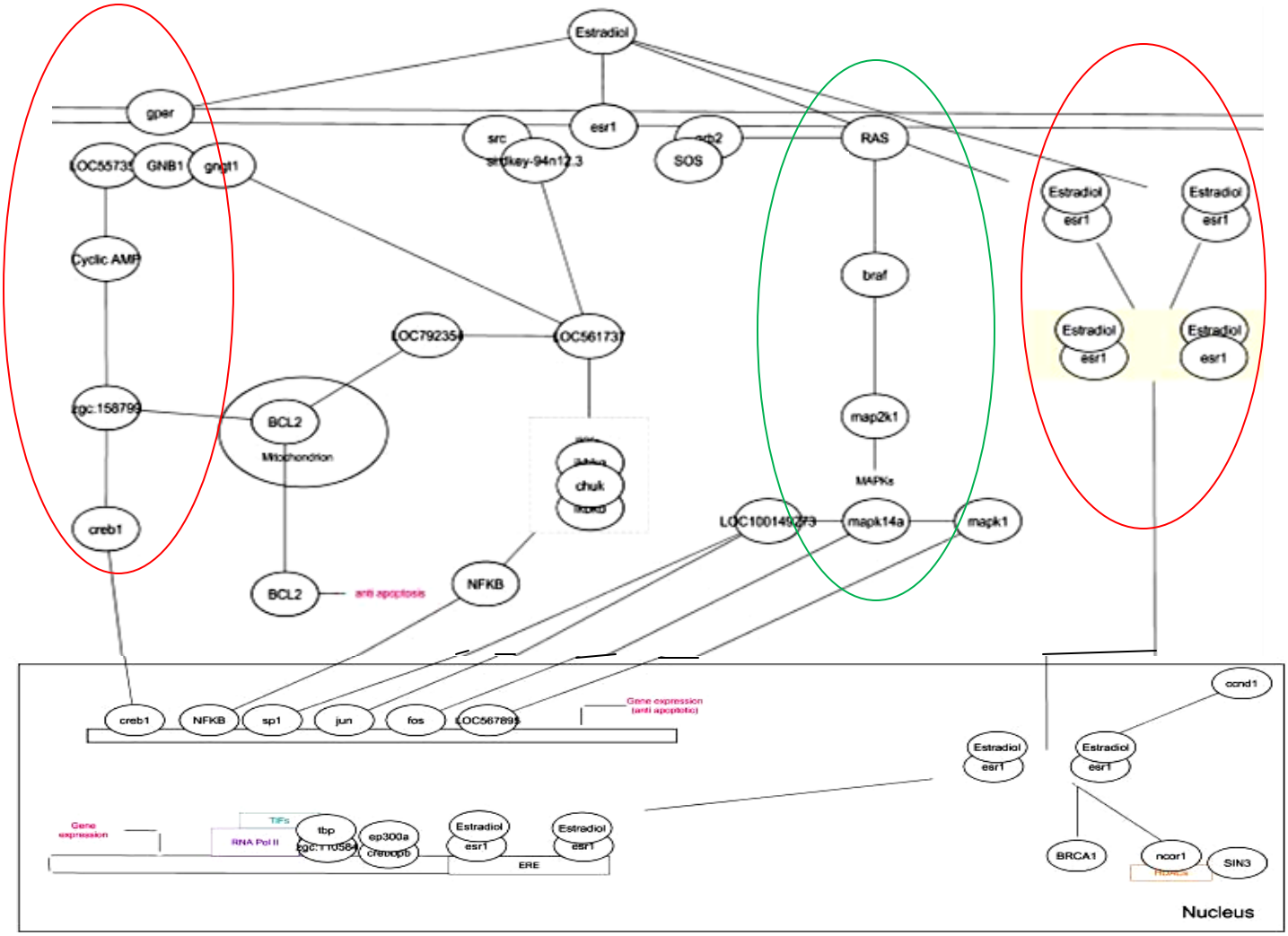

Figure 2.21: Schematic representation of Estrogen signaling genes. Green circle represents the less operative pathway while red circles represents possible operative pathway.

Discussion:

The increase in the spray of agrochemicals has resulted in elevation of toxicity in the environment that has end result in bioaccumulation from one trophic level to the other, ultimately affecting the humans (Ribeiroa *et al.*, 2005; Guo *et al.*, 2008; Singh and Singh 2008). Teleost are one of the remarkable models of studies, ranging from developmental to toxicology (Fujimura and Okada 2007). In the present study, *O.mossambicus* was taken as the model organism with the aim to find the endocrine disrupting properties of the agrochemicals. Key genes were studied to check the altered physiology on exposure of the widely used agrochemicals in the state of Gujarat. An attempt is made to identify relevant perturbations in HPG, HPI and HPT axis genes on exposure of agrochemicals, to know the impact on key regulatory molecules within all the three.

The neuroendocrine system of the HPG axis regulates reproduction in vertebrates and can be influenced by chemicals, therefore affecting the reproductive system. Neurotoxic environmental contaminants recognized as endocrine-disrupting chemicals (EDCs) have aroused considerable interest in the field of neuroendocrinology (Panzica *et al*. 2005; Gore 2008a, b). Among these pollutants, some of the selected pesticides are considered to be hazardous because they are very persistent, non-biodegradable, and ubiquitously found in the environment. In vertebrates, the hypothalamus represents a master regulator of homeostasis and is the critical nexus between the nervous and endocrine systems. The hypothalamus mediates responses to homeostatic imbalance mainly through regulation of the pituitary gland, which, in turn, produces hormones that are able to affect systemic change in the gonads. The central role of HPG axis makes it particularly susceptible and sensitive to perturbation by a variety of environmental contaminants. Chemical disruption of the HPG axis often results in modifications of circulating hormones, leading to an inability to mitigate environmental stress, as well as, directly alters the reproduction and development, which produce population level impact on fish (Kidd *et al*., 2007 and Miller *et al*., 2007).

Teleost particularly *O.mossambicus* are known to have three isoforms of *GnRH (GnRH-I ,GnRH-II ,GnRH-III)* that are distributed in various tissues, till date *GnRH-I* is identified to regulate HPG (Nocillado and Elizur 2008; Maruska and Fernald 2011; Sempere *et al*., 2012) and that GnRH I neurons are known to be regulated by kiss 2 neurons of discrete nuclei of

hypothalamus in some teleost (Oakley *et al.,* 2009), thus intimately regulating each other (Parhar *et al.,* 2004; Clarkson *et al* .,2010). In the present study, of all the agrochemicals exposed, the expression of GnRH-I was found to be up regulated in MN, CZ and IMI exposed group with a significant down regulation except in PE exposed group compare to control. In fish GnRH-I is known to be regulated by negative feedback of circulating hormonal levels through its receptor located in hypothalamus and pituitary (Weltzien *et al*., 2004, Zohar *et al*., 2010). PE being a herbicide belonging to the group of Sulphonyl urea has elevated the levels of hormones (Estradiol and Testosterone Chapter I) which directly confirms the mechanism, suggesting that the GnRH-I fibers present in the pituitary, probably through its primary hypophysiotropic role has a strong correlation between GnRH-I expression in brain and gonadal activity. Thus, our results are in agreement with the results of Khan and Thomas(2001) where atlantic croaker was exposed to aroclor 1254, Piazza *et al.,* (2011) where fish larvae was exposed to endosulphan.

Kiss2 mRNA expression was also found to be significantly up regulated under the exposure of MN, IMI and CZ, implying that it is exerting its effect by up-regulating GnRH-I and Kiss2 neurons. Furthermore, MN exhibited the maximum alteration in the Kiss2 gene expression pattern, possibly due to the nature of MN, which is an amalgamation of trace metal ions (Zn^{2+},Fe^{2+},Cu^{2+},B^{+},Mn^{+}) proposing the synergistic or individual action of metal ions (Brian *et al*., 2005, Correia *et al*., 2007; Filby *et al*., 2007; Finne *et al*., 2007; Zhang *et al*., 2009; Mebane *et al.,* 2012; Sadekarpawar *et al.,* 2015). IMI belongs to neonicotinoid group, is known to exert its effect by blocking the actylcholinesterase activity in brain. In the present study, the increase in GnRH-I and Kiss2 proves the genotoxic potential of IMI in altering the activity of gonads and thereby on reproduction apart from its usual mode of action (Andersen *et al.,* 2004; Kitahashi *et al*., 2009;Desai and Parikh 2013; Bharadwaj and Sharaf 2014; Gibbons *et al.,* 2015; Crosbya *et al.,* 2015; Ansoar-rodriguez *et al*., 2015; Dang *et al.,* 2015). However, these responses, although critical to understanding the mechanism of agrochemicals, need to be fully examined and integrated in a broader system to support more reliable prediction of it. To understand the probable downstream signaling mechanism,

Phosphotidol Inositol (PI) and Protein kinase C (PKC) are important factors in downstream signaling pathway. Inhibition of PKC has been reported to block the gene activity, proving its role at transcriptional level (Ghosh and Ray 2012). GnRH activates multiple signal transduction

pathways such as Ca^{2+} and cAMP signaling through binding to GnRH-R (Ruf *et al*., 2003; Millar *et al*., 2004), which stimulates phospholipase C to generate inositol trisphosphate and diacylglycerol. Increases of these signaling messengers lead to activation of protein kinase C (PKC) and also an increase in intracelullar Ca^{2+} concentration. These two secondary signal mediators are involved in GnRH-induced GTH release and synthesis (Klausen *et al*., 2002; Ando and Urano 2005). An up regulation of PKC gene (IMI, MN and CZ) and down regulation (PE), thus indicate the multiple signaling pathway through which the agrochemicals are altering the expression. Our results are parallel with earlier reported work of Yaron *et al.,* 2003; Ando and Urano 2005; levavi-Sivan *et al.,* 2010 where they have reported the molecular mechanism of divergent physiological strategies of reproductive success in various teleost.

Kisspeptins are a group of peptides that stimulate GnRH release and are required for puberty and maintenance of normal reproductive function. Studies in teleosts have revealed the presence of multiple kisspeptin forms (Kiss1, Kiss2) in the brain. It has been suggested that there is a double site of Kisspeptin action in the brain, either in the hypothalamic-hypophyseal region or in the median eminence, an area located outside the blood brain barrier (Nocillado *et al.,* 2008). The important role of Kiss 1 has also been established to regulate gonadotropin secretion, confirming the pivotal role in regulation of reproduction (Akazome *et al*., 2010). Neurons expressing kissspeptins are key players in controlling the cyclic activity of the reproductive axis, possibly by activating GnRH neurons (Roa and Tena-Sempere 2007; Tena-Sempere 2010; Escobar *et al*., 2013). Expression of kisspeptins and its receptors exhibits interspecies variation (Escobar *et al*., 2013). In line of this, the results of the present study where, mRNA expression and western blot analysis has confirmed the expression of kiss 1 in *O.mossambicus* for the first time. A significant down-regulation of kiss 1 under PE exposure, suggests its non-essential role for reproduction (Servili *et al*., 2011; Ogawa *et al*., 2012 Tang *et al*., 2015). However, the exact mechanism by which it happens is still illusive. Immune-histochemical and cloning studies will be able to shed more light on the same. These results thereby suggest that of all the agrochemicals; PE, IMI and MN are capable of interfering with kiss 2 and GnRH system thereby altering the HPG axis.

The endocrine system functions due to the presence of hormone interaction with cognate receptors (Casals-Casa and Desvergne 2011). The receptors are classified as membrane-bound receptors (GtH Ir and GtHIIr) and nuclear receptors (ER I, ER II, ARI and AR II). The

membrane-bound receptors typically bind peptide hormones, while the nuclear receptors bind small lipophilic hormones including the sex steroids. *GnRH-I* upon activation acts on cells of anterior pituitary, thus initiating the release of GtH-I (FSH like) and GtH-II (LH like) peptides. This in turn binds to its receptor (GtH-Ir, GtH-IIr) present either on gonads (Yaron *et al.,* 2003; Chen and Fernald 2008; Maruska and Fernald 2011). Receptor profile of *GtH-Ir* and GtH-IIr was found to be up regulated on IMI exposure; whereas CZ exposure resulted into up regulation of GTH Ir ,whereas MN exposure produced an up regulation of GTH IIr. An upregulation in GtHs suggest either an operation of kiss 2 or PKC mediated pathway leading to an increase in GtHrs culminating into either vitellogenesis/spermatogenesis in the gonads (Yousefian and Mousavi 2011). Our results are in accordance with earlier reports in zebra fish (Ho *et al*.,2003; Kitahashi *et al*., 2009).

Many EDCs are small lipophilic compounds and are capable of interacting with nuclear receptors (Casals-Casa and Desvergne 2011). This often causes changes in gene expression of the hormones involved in steroidogenesis. Agrochemicals in the form of EDCs often target receptors as either agonists, which mimic a naturally occurring hormone or antagonists, which block the action of a naturally occurring hormone. These can affect receptors throughout the HPG-axis. Some of these chemicals disrupt a variety of hormone regulated physiological pathways, including reproductive responses mediated by ER and AR in vertebrates (Bowman *et al*., 2002; Larkin *et al*., 2003; Johnston 2013;Nelson *et al*., 2013; Baker and Hardyman 2014). Both estradiol and androgens have been shown to directly regulate pituitary expression of gonadotropin subunit in several fish species (Huggard-Nelson *et al*., 2002). *ER-I* and *ER-II* analogous to mammalian estrogen receptors α and β (Nagler *et al.,* 2007; Guiguen *et al.,* 2010; Nelson and Habibi 2013) were studied in ovary, brain and testes. Among all the agrochemicals exposed, there was significant up regulation of *ER-I* in ovary, brain and testes of IMI and MN exposed groups, possibly governing the action by some downstream signaling mechanism (Selin *et al.,* 2009), confirming the endocrine disrupting action of these chemicals. *ER-II* did not show same pattern of regulation as CZ and PE exposure resulted in a significant up regulation of *ER-II* mRNA in brain and ovary, while IMI accredited the higher expression of *ER-II* only in brain tissue. CZ being the mixture of cymoxanil and mancozeb, has resulted into constitutive receptor activation leading to its up regulation (Villeneuve *et al*. 2009; Coumailleau *et al.,* 2015), which may be due to its mimicking action as that of estrogen. Apart from the conventional studies done

on various groups of herbicide (Upadhyay *et al.*, 2014), very few studies are accounted for the negative effects of PE on any organism. PE which belongs to the group of sulfonylurea too expressed the parallel effect as that of CZ, suggesting its mimicking role to that of estrogen which was well supported by an increase in plasma level of 17- β estradiol (chapter I). Our result are parallel to the result of Kim *et al.*,(2014); Servili *et al.*, (2011). In teleost estrogen signaling is mediated through three ER subtypes and each subtypes is likely to show differential responses (cAMP, MAPK, directly activation of transcription factors) to ligands which ultimately results in a deleterious effect on those pathways to affect the physiological functions. In the present study, there was a down regulation of MAPK suggestive of activation of either of the two canonical pathway (Cabas *et al*.,2013; Tohyama *et al.*, 2015).However, full elucidation of mechanistic molecular pathways by which agrochemicals are modulating the estrogen signaling, requires a better understanding of distinct roles of each ER subtypes.

Compounds that inhibit androgen-signaling can act through several modes of action: (1) competitively binding to the androgen receptor (AR), thus inhibiting the transcription of androgen-dependent genes (Villeneuve *et al*., 2007; Martinovic *et al*., 2008; Hatef *et al*., 2012 and Golshan *et al*., 2014); (2) modifying the production of androgens through the inhibition of rate-limiting genes and enzymes involved in steroidogenesis; or (3) increasing the degradation of androgen precursors and testosterone (T) in the testes or liver. In all three cases, the compound(s) disrupts one or multiple biologic pathways along the HPG (Crago and Klaper 2012).In the present study, there was an up regulation of AR I and AR II on exposure of all the agrochemicals, however significant up regulation was noticed only in CZ and IMI exposed fish probably impairing the gonadal function which has lead to 17 B estradiol (E2)/ 11-keto testosterone (11kt) imbalance (Chapter-I). Our results are in accordance with the earlier reported work of Kubota *et al*., 2003; Loutchanwoot *et al*., 2008; Martinovic *et al*., 2008; Eustache *et al*., 2009;Hatef *et al*., 2012; Golshan *et al*., 2014, 2016 in various teleost fish on exposure of different EDCs.

Physiological responses of fish to environmental stressors have been grouped broadly as primary and secondary. Primary responses, which involve the initial neuroendocrine responses, include the release of catecholamines from chromaffin tissue (Gundersen *et al*., 2000; Barton 2002; Hontela *et al*., 2008), and the stimulation of the hypothalamic-pituitary-interrenal (HPI) axis

culminating in the release of corticosteroid hormones into circulation (Barton *et al.*, 2002 and 2005; Martínez-Porchas *et al*., 2009). Secondary responses include changes in plasma and tissue ion and metabolite levels, hematological features, and heat-shock or stress proteins (HSPs), all of which relate to physiological adjustments such as in metabolism, respiration, acid-base status, hydro-mineral balance, immune function and cellular responses (Portz *et al*., 2006; Martínez-Porchas *et al*., 2009). Additionally, tertiary responses occur, which refer to aspects of whole-animal performance such as changes in growth, condition, overall resistance to disease, metabolic scope for activity, behavior, and ultimately survival (Barton 2002; Husen and Sharma 2014). Much of our present knowledge about physiological responses of fish to stress has been gained from studying the primary responses of the brain-chromaffin and HPI axis to stressors and the subsequent or secondary effects associated with neuroendocrine stimulation on metabolism, reproduction, and the immune system (Barton *et al*., 2000; Davis 2004; Hontela 2005). Comparatively less information is available on gene expression of fish to environmental perturbations associated with natural or anthropogenic stressors, particularly water-borne contaminants like agrochemicals.

In all vertebrates, gluco-corticosteroids play a key regulatory role in stress responses, growth, metabolism, reproduction and immunity (Stolte *et al.,* 2008). Studies have investigated the role of cortisol with GR and/or MR in fish osmoregulation, primarily through pharmacological approaches; however, some of the results are conflicting. Genome duplication event occurs in teleost fish (Jaillon *et al.,* 2004), leading to two distinct GR genes (Bury *et al*., 2003; Greenwood *et al*. 2003; Bury & Sturm 2007; Stolte *et al.,* 2008; Alsop *et al*.,2008). In the present work, focus was mainly on the GR, as it has high affinity to bind its peptide cortisol (Basu *et al*. 2003**;** Shelly *et al.*,2013). Indeed, several studies have shown that the xenobiotics disrupt cortisol and its receptor response to stress by targeting multiple sites along the HPI axis, including impaired steroidogenesis and brain glucocorticoid signaling (Aluru *et al.,* 2004; Hontela, 2005; Vijayan *et al.*, 2005; Aluru and Vijayan, 2006).In the present study, tissue specific receptor expression was studied under the exposure of agrochemicals, among which, significant damage was encountered by IMI, which down regulated the mRNA of GR in all the organs followed by CZ in ovary and testis only. The exposure of IMI and CZ resulted in sensitization of cortisol receptor and may be its peptide (cortisol), substantiating the receptor down regulation probably due to its self regulation (Sathiyaa and Vijayan 2003). Thus, the mechanism that may be operative is the

increased in the cortisol peptide which binds to its receptor leading to activation of other growth regulated transcriptional factors and thus maintaining the physiological stress caused by the agrochemicals (Gravel and Vijayan 2006). Moreover, earlier studies have been already established the primary, secondary and tertiary responses of these agrochemicals (Upadhyay *et al*., 2014; Sadekarpawar *et al*., 2015, Patel *et al*., 2016)

Over the past 20 years, the potency of endocrine-disrupting chemicals (EDCs) has attracted attention in numerous ecotoxicological studies (Matthiessen 2003; Blanton and Specker 2007; Hotchkiss *et al*., 2008; Kloas *et al*., 2009; Bernanke and Köhle 2009). So far, the main focus of EDCs was clearly on effects on reproductive biology (Gray *et al*.,2003), but the awareness of potential risks by thyroid system-disrupting chemicals is increasing. In common with the reproductive steroid hormones, the synthesis and release of the thyroid hormones is under the control of a central Hypothalamus-pituitary-thyroid (HPT) axis (Scholz and Mayer 2005; Eales, 2006, Blanton and Specker, 2007 and Zoeller *et al*., 2007) .Thyroid stimulating hormone is released by pituitary is the primary physiological regulator of thyroid gland function, stimulating thyroid hormone synthesis/ release and exerting trophic effects on thyroid tissue. In teleost two isoforms of TSHr (TSH-αr and TSH-βr) have been established (Kopp 2001; MacKenzie *et al*., 2009: Opitz *et al*., 2011). In line of this, TSH-βr expression pattern was studied in thyroid tissue under the exposure of agrochemicals, where IMI exposure resulted in a significant increase, while PE, CZ and MN exposed groups showed non-significant down regulation. Hence from the present study one can conclude that the agrochemicals have resulted into an overall alteration into HPT axis thereby affecting the overall synthesis or release of the thyroid hormones. Our results are in agreement with the work of Ghisari *et al*., (2015) where they have checked the effect of 13 pesticides on thyroid profile *in-vitro* and Rossi *et al*., (2007) ; Picchietti *et al*., (2009) who have reported interference of DDT metabolites with TSH receptors on thyroid follicular cells.

To analyze interactions between the specific and non-specific genes involved at the transcriptional level an attempt was made to interconnect with the help of Cytoscape software , pathway common and wiki pathways was used which helped in understanding the possible role of the association of genes with the target genes. Normal pathway which gets activated by GnRH I is through specific receptors, that will alter the hormonal titer through PKC pathway. By

incorporating the data in the pathway common software and wikipathways it was found that GnRH I was seen to be interacting with additional transcriptional factors ZEB1 and EN1 Suggesting the possible role of ZEB1 in modulating the GnRH I expression culminating into an increase/decrease in the downstream signaling molecules leading to a alteration in hormonal titers. Cytoscape software also showed interaction with Prolactin releasing hormone (PRL) and Thyroid releasing hormone (TRH). The neurons in the hypothalamus are known to be intimately associated with each other i.e thyrotrophs, lactotrophs and gonadotrophs. Hence, the possible reasons for its interaction with TRH and PRL are difficult at this point of time as co-imunoprecipitation studies were not done.

The pathway common software revealed that GtH-II had association with 6 different genes and GtH-I was found to be associated with the genes of steroidogenesis, chemokines and other transcriptional factors. As far as GtH II is concerned CYP19A1 (aromatase) was the only gene directly associated with hormonal alterations and is known to convert testosterone into estrogen proposing it's role in alteration of the hormones on agrochemical exposure. Other genes however, may probably have its role in metabolism. On the other hand CYP11A1 (StAR protein) was found to be associated with GtH-I suggesting a supportive role of these genes in mediating the alterations in hormonal titers on agrochemical exposure. Estrogen through its receptor is known to mediate downstream signaling by three canonical pathways. In the present study insignificant alterations in MAPK2, thus suggest the operation of other two pathways (cAMP and or direct diffusion) and their respective genes in the downstream signaling in mediating the alterations in hormonal titers on agrochemical exposure. Thyroid axis genes study revealed that TSH was associated with its releasing factor (TRH) and other transcriptional regulators (FOXL, GATA1 etc) and it was only TRH which was associated with CYP26A1, proposing it importance in downstream signaling probably having the role in mediating the response of the hormonal titers. GR signaling governs many metabolic pathways through more than 100 genes. The data of the present work was applied to Cytoscape and pathway commons, which resulted into exposure of role of SRDVA I and II which is known to convert testosterone into its more potent form. Thus, aiding in combating the agrochemical stress and maintaining its reproductive potency.

Conclusion:

So from the present work it can be concluded, agrochemicals exposure invoked alterations in the expression of genes associated with HPG, HPI and HPT axis. A strong link is thus determined between the measured up regulation/down regulation of specific genes and indicates the potential of using gene expression in toxicological studies as markers. Furthermore, the result of this study illustrates the potential risk of agrochemicals to non-target organisms in aquatic environment and indirectly to human health. However, immune-histochemistry, micro array and cloning of the downstream genes will provide insights into better understanding of the mechanisms governing the effect of agrochemicals on the canonical pathways of the three axis.

Gel Plates

Below are some of the gel plates of representative genes of the three axis.

***GnRH-I (180bp)*:**

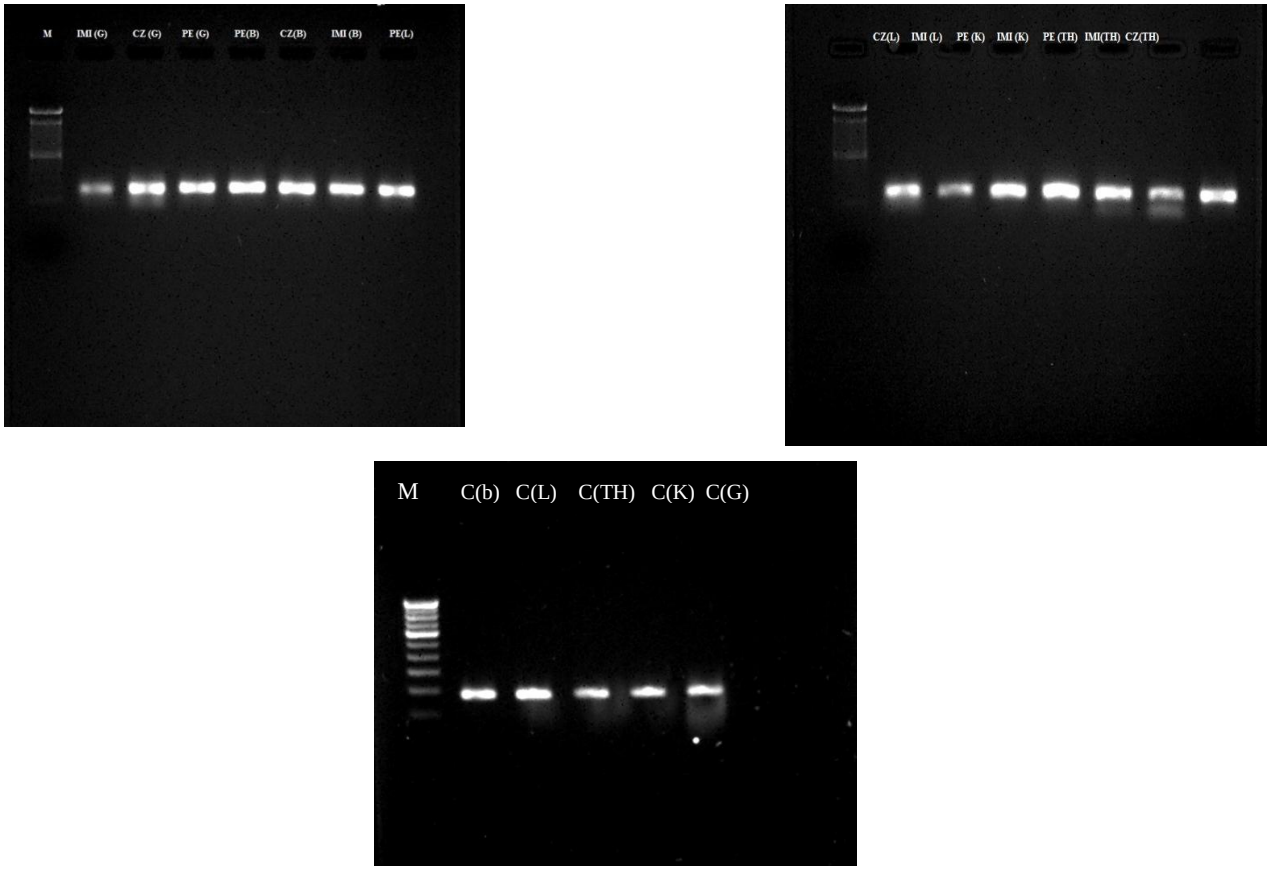

Figure 2.22: 2% agarose gel image of GnRH-I of control, CZ, PE, IMI, and MN.

Kiss 1 (156bp):

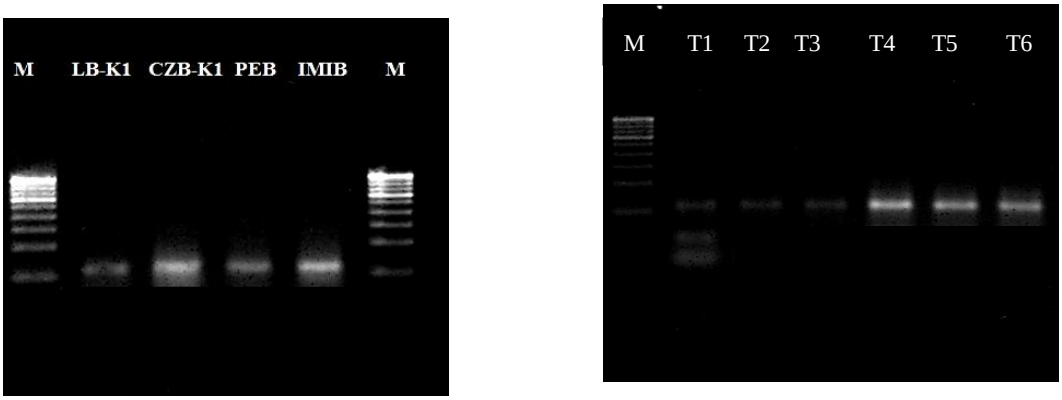

Figure 2.23: 2% agarose gel image of kiss 1 of control, CZ, PE, IMI, and MN. T1-T6 represents different temperature of control samples

Kiss 2 (214bp):

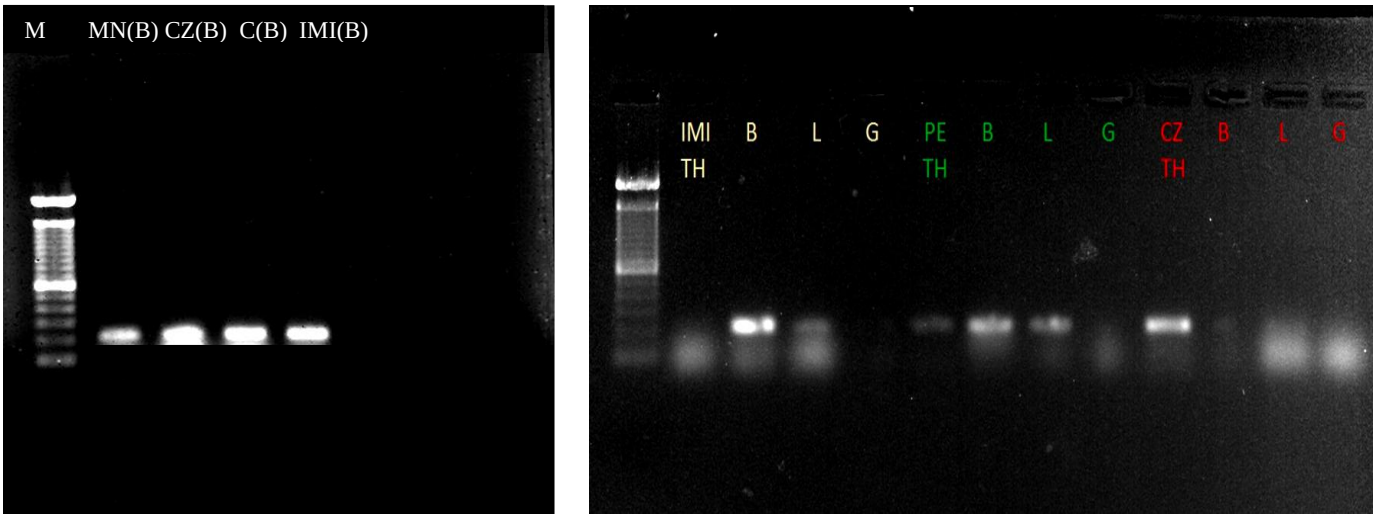

Figure 2.24: 2% agarose gel image of kiss 2 of control, CZ, PE, IMI, and MN.

GtH- Ir (276bp):

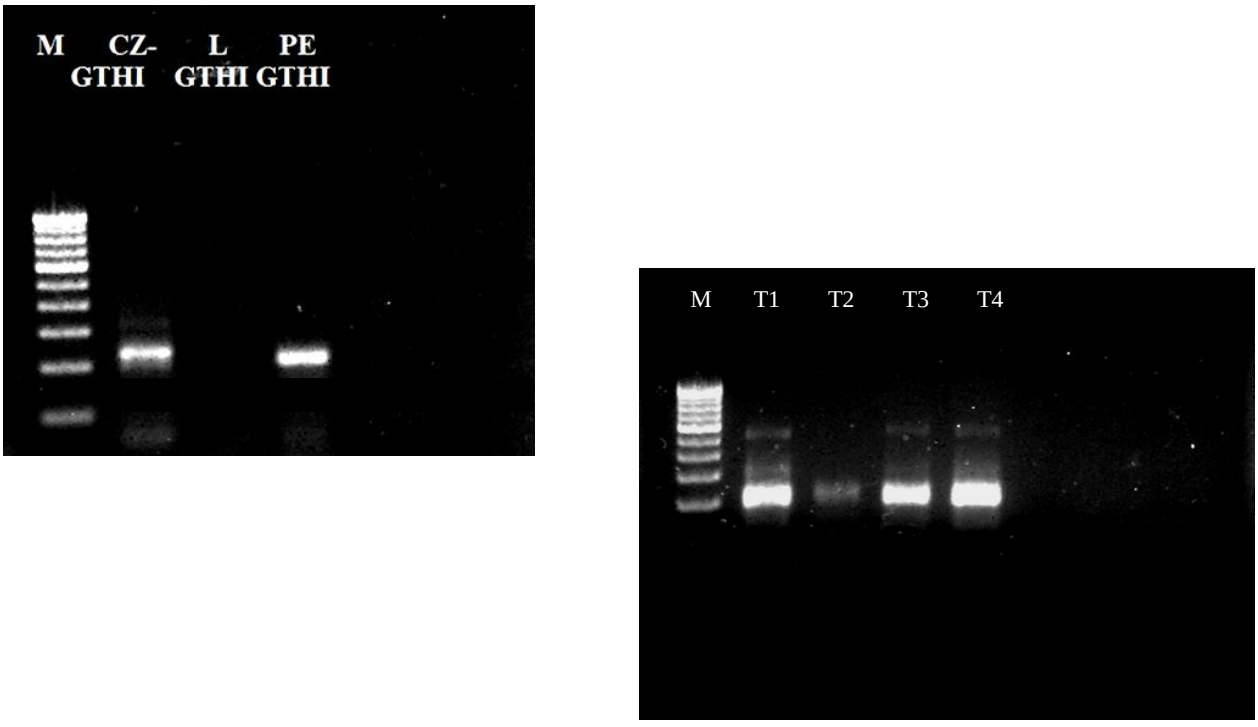

Figure 2.25: 2% agarose gel image of GtH-Ir of control, CZ, PE, MN. T1-T4 represents different temperature of control samples

ER-I (85 bp):

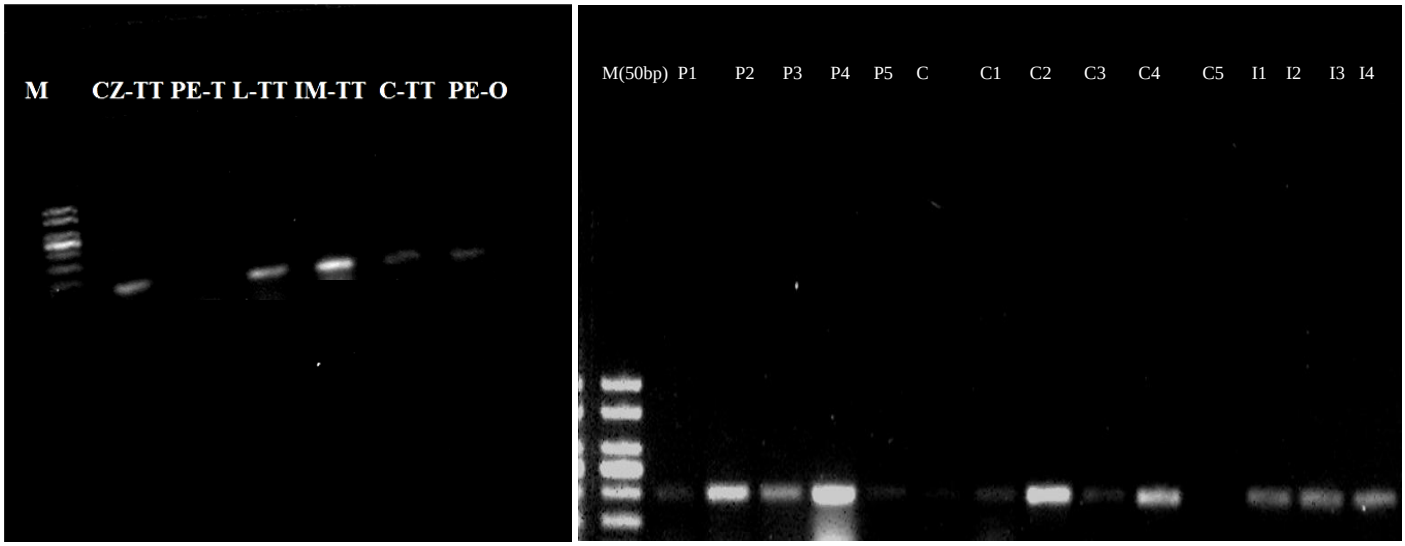

Figure 2.26: 2% agarose gel image of ER-I of control, CZ, PE, MN. P1-P5,C1-C5 and I1-I4 represents different samples.

ER-II (198bp):

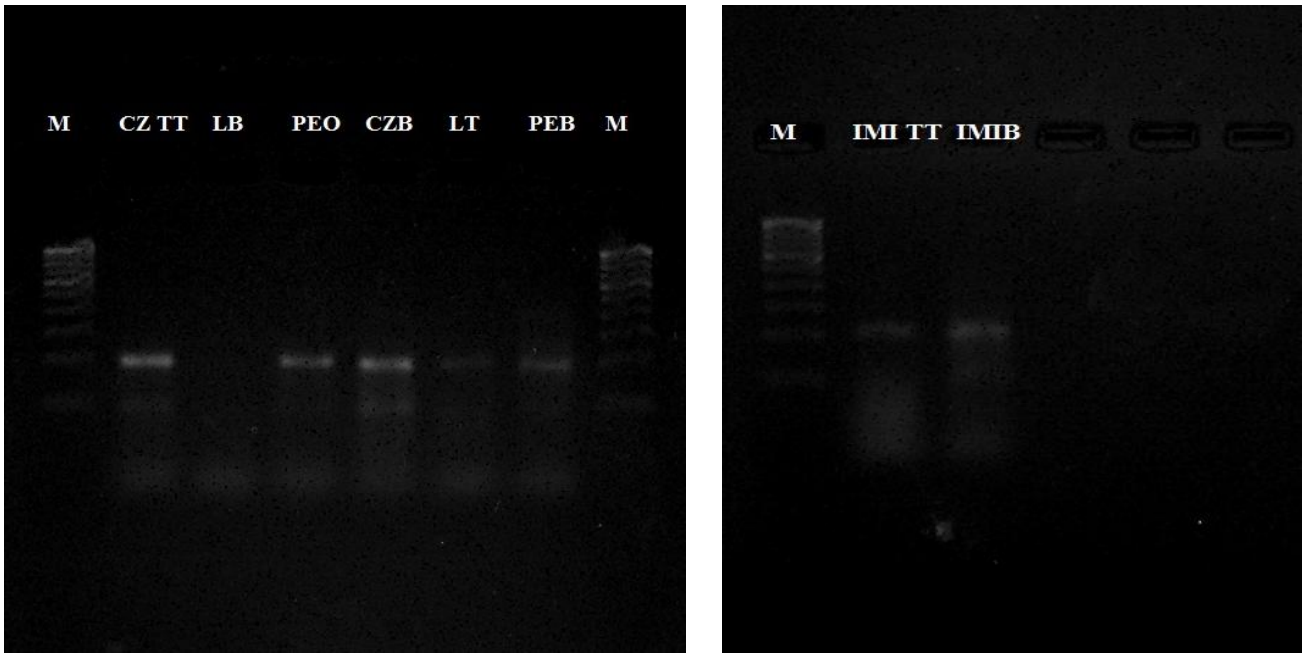

Figure 2.27: 2% agarose gel image of ER-II of different samples.

AR-I (73bp):

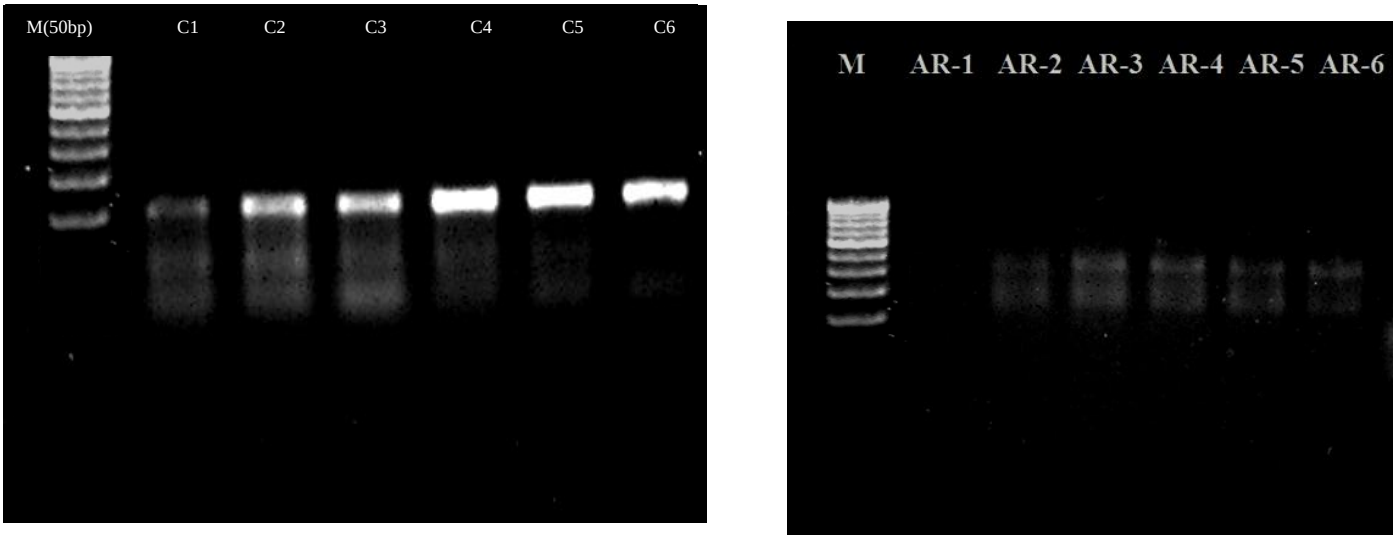

Figure 2.28: 2% agarose gel image of AR-I of different samples.AR1-6 are standardization of different samples at different temperatures.

GR(113 bp):

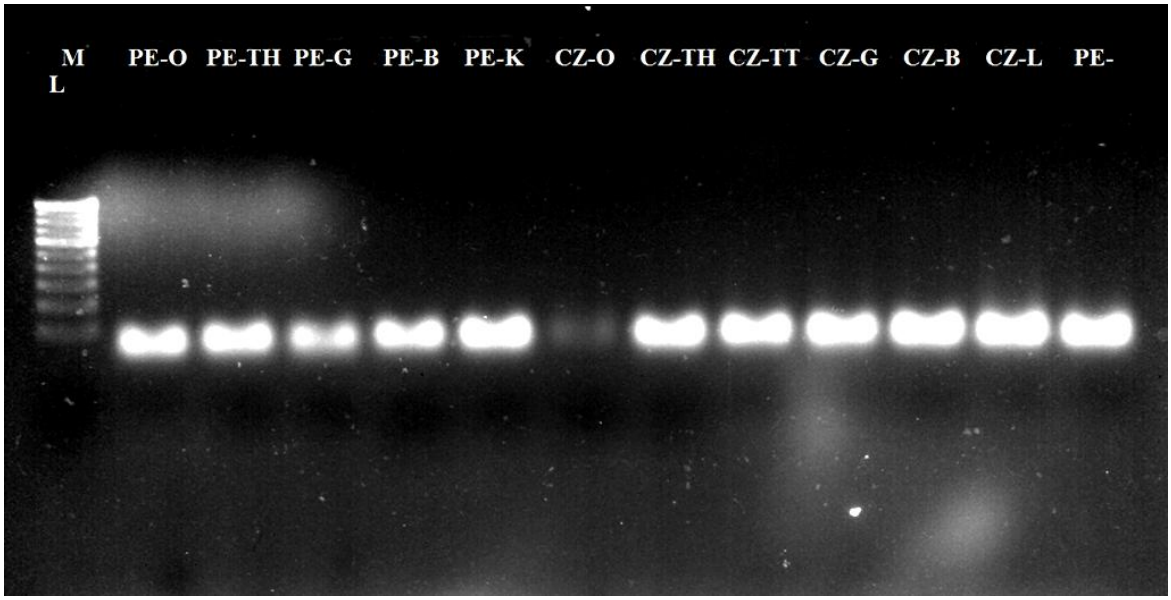

Figure 2.29: 2% agarose gel image of GR of different samples.

PKC (242bp):

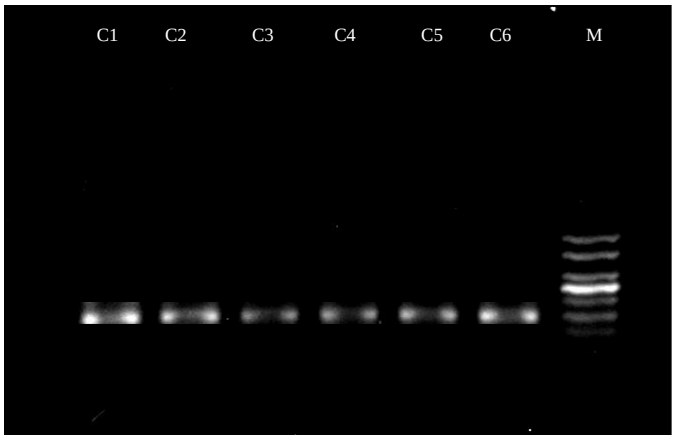

Figure 2.30: 2% agarose gel image of PKC of different samples (C1-C6).

18srRNA (102bp):

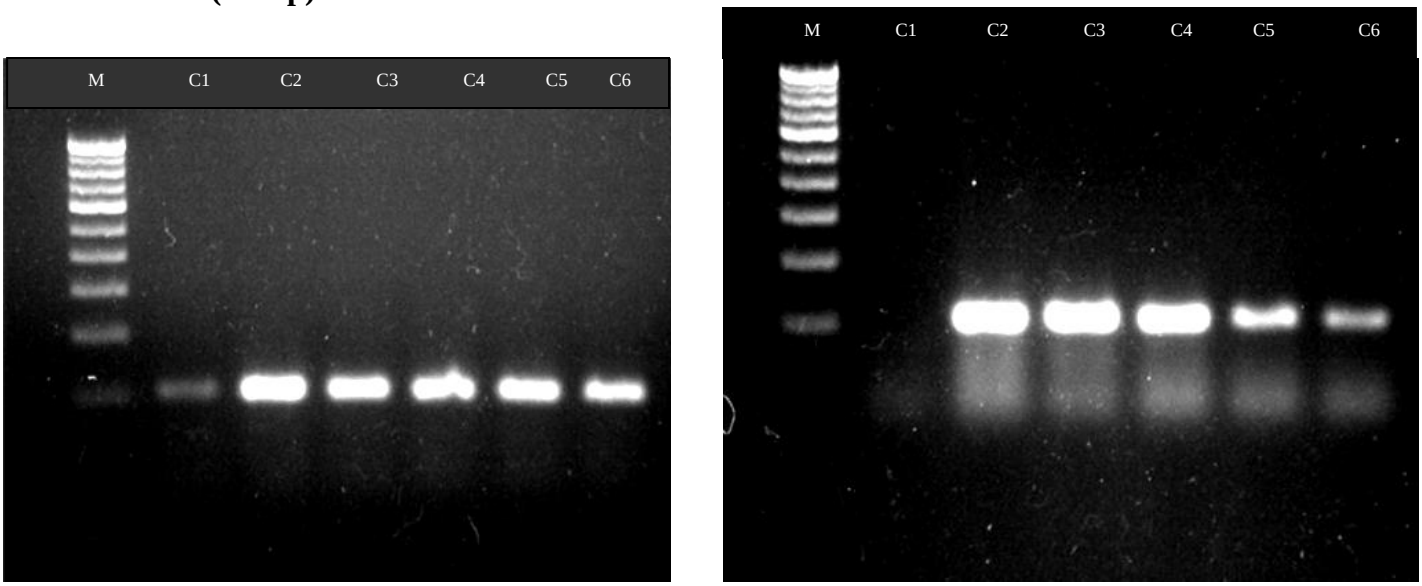

Figure 2.31: 2% agarose gel image of 18s rRNA of different samples (C1-C6).